Role of CCR5 in the cardiovascular complications of renal disease and diabetes:

insights from studies on CCR5Δ32 genotype

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insights from studies on CCR5Δ32 genotype

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Contents

Chapter 1

INTRODUCTION AND OUTLINE

Introduction

By the end of the 20th century atherosclerotic cardiovascular disease was expected to be eliminated due to adequate treatment of the classic cardiovascular risk factors, hypercholesterolemia and hypertension. However, nowadays cardiovascular disease is expected to be the main cause of morbidity and mortality owing to increasing prevalence in developing countries and rising incidence of obesity and related diseases like diabetes, hypertension, dyslipidemia and chronic kidney disease in the western world.[1] Especially in patients with type 2 diabetes and end-stage renal disease (ESRD) cardiovascular disease is a prominent cause of mortality.[2-4] In particular in the latter patient group therapies proven to be successful in the general population have thus far been disappointing.[5-7] This underscores the importance of unravelling the atherosclerotic process, in particular in renal patients, to ultimately discover novel targets for prevention and treatment.

Atherosclerotic lesions

The atherosclerotic process is characterized by arterial lesions that progress from endothelial dysfunction to an initial fatty streak towards an unstable, vulnerable plaque in the arterial vessel wall. The process responsible for this is called 'the response to injury hypothesis' described by Ross and Glomsed.[8] This process is initiated by activation and dysfunction of endothelial cells. Both environmental factors, like hyperlipidemia and genetic factors play a crucial role in this process (gene-environment interaction). The endothelial dysfunction together with expression of adhesion molecules and release of cytokines and chemokines increases vascular permeability and allows monocytes and T-cells and other inflammatory cells to migrate into the subendothelial space.[4, 9-12]

The fatty streak is an early atherosclerotic lesion caused by the local uptake of lipids by endothelial cells, particularly at sites of hemodynamic strain. In the intima, these lipoproteins are modified into oxidized lipoproteins, which cause a local inflammatory response again leading to platelet and leucocyte adhesion. Monocytes invading the fatty streak differentiate into macrophages that take up excess lipids, a process that eventually causes them to differentiate into foam cells. Accumulation of additional inflammatory

cells leads to atherosclerotic plaque formation. The more advanced stable plaque (atheroma) consists of a thick fibrous cap with high collagen and smooth muscle cell content and a lipid core that in turn is constituted by foam cells, debris and lipid droplets. The presence of an intact advanced plaque may lead to a stenotic obstruction of the blood vessel and hereby leading to angina. Moreover, mechanisms that largely remain unknown but are clearly related to macrophage infiltration may render a stable plaque unstable and prone to rupture. Plaque rupture results in exposure of the plaque's prothrombotic core content and leads to massive local blood coagulation and formation of a thrombus. Such thrombosis may lead to obstruction of blood vessels and hereby to infarction.[4, 11]

Inflammation, end stage renal disease, diabetes and atherosclerosis

It is now widely recognized that inflammation plays a crucial role in the development of atherosclerosis.[4, 13, 14] This chronic inflammatory condition is, at least in part, responsible for the elevated overall and cardiovascular mortality seen in type 2 diabetes.[3, 4, 15] Also in ESRD a persistent inflammatory state is a well know risk factor for morbidity and mortality, both cardiovascular and non-cardiovascular.[16-19]

The immune response seen in atherosclerosis can be separated into an innate immune response involving expression of adhesion molecules, chemokine release and monocyte/ macrophage and natural killer (NK) T cell recruitment and an adaptive immune response involving B cells and antigen specific T cells (Figure 1 and 2).[1, 20-23] The B and T cells respond to antigens presented by antigen presenting cells (dendritic cells, B cells and macrophages). Numerous candidate antigens have emerged, both microbial and self-antigens, but their contribution during atherogenesis remains unclear.[20] Different T cell subclasses are involved.[24] Both CD4+ and CD8+ T cells are found in human lesions but CD4+ cells dominate.[20, 23, 24] CD4+ T cells can differentiate into different subsets, like T helper 1 (Th1) cells, T helper 2 (Th2) cells, T helper 17 (Th17) cells and regulatory T cells. The principal cytokine of Th1 cells is IFN-γ and is produced by the majority of all T cells in atherosclerotic plaques. Th1 cell activation also leads to TNF-α secretion. IFN-γ and TNF-α are thought to play a pro-inflammatory, pro-atherosclerotic role.[20, 21, 24, 25] Th2 cells and the cytokines IL-4, IL-5 and IL-10 play, at least in part an anti-inflammatory, anti-atherogenic role.[20, 24, 25]

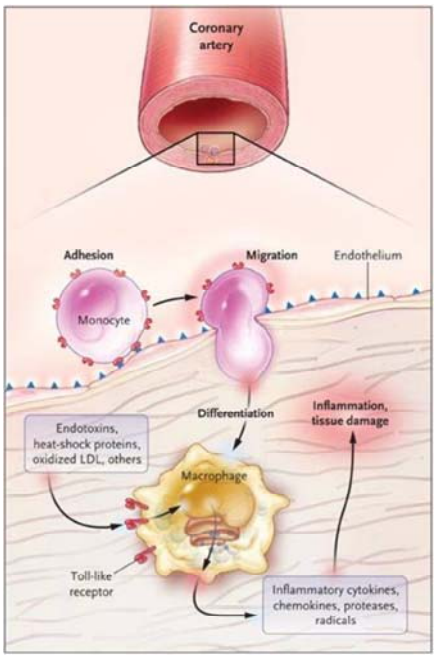

Figure 1: Monocyte recruitment through the activated endothelium and differentiation into macrophages. These macrophages release cytokines, chemokines and other inflammatory cells leading to inflammation and tissue damage (reprint with permission, N Eng J Med 2005;352-1685-95).

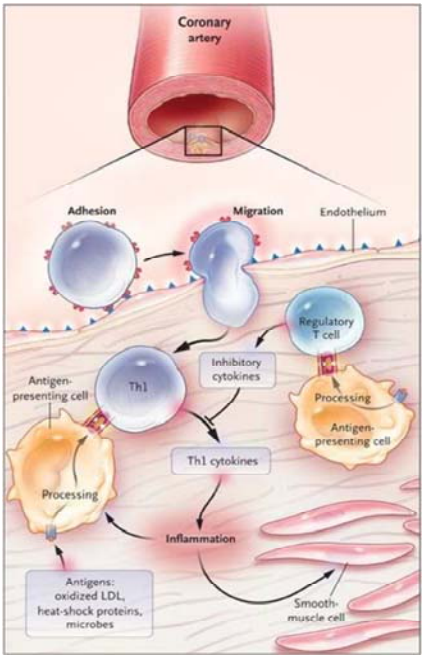

Figure 2: Antigens presented by macrophages and dendritic cells trigger activation of antigen specific T cells. Most of the activated T cells produce Th1 cytokines leading to inflammation. Regulatory T cells modulate this process (reprint with permission, N Eng J Med 2005;352-1685-95).

As with CD4+ T cells, CD8+ T cells can differentiate to T cytotoxic (Tc)1 or Tc2-cell subsets, secreting predominantly Th1 or Th2 cytokines respectively.[26] The role of IL-17, which is produced by Th17 cells, in atherosclerosis is not yet clear.[25, 27] Regulatory T cells, especially regulatory T cells type 1 are thought to play a central role in counteracting disease initiation and progression.[20, 28] The effects of B cells and their antibodies seem to depend on their antigen specificity.[20, 29]

In this process of inflammation and atherosclerosis different chemokines play an important role.[12, 30] Chemokines can be classified into four major categories (C, CC, CXC and CX3C) depending on the arrangement of their N-terminal cysteine residues. Chemokines bind to and activate specific G

protein coupled receptors, chemokine receptors, present on the surface of leucocytes, dendritic cells, endothelial cells and smooth muscle cells resulting in specific cellular functions such as migration (chemotaxis), firm adhesion to the vessel wall and/or transmigration through the vascular endothelium. The involvement of chemokines and chemokine receptors suggests that these 'players' may provide novel targets for therapeutic intervention in atherosclerosis-related diseases. From the chemokine family CC chemokines have been widely implicated in atherosclerosis and also CC chemokine blockade has been found to reduce atherosclerosis in ApoE knockout mice.[31] In particular there is evidence for important and distinct roles of CCL2/CCR2, CCL5/CCR5, CX3CL1/CX3CR1 and CXCL8/CXCR2 in atherosclerosis.[11, 32]

CCR5

One of the chemokine receptors with a possible role in atherosclerosis is the CC-chemokine receptor 5 (CCR5). CCR5 is expressed on T cells, monocytes/macrophages, smooth muscle cells and endothelial cells.[33, 34] These cells are involved in the chronic inflammatory state that is observed in insulin resistance, type 2 diabetes, atherosclerosis and uremia.[4, 10, 15, 17] Several genetic polymorphisms have been described for CCR5. The CCR5Δ32 genetic variant is located on the chromosome 3p21 and consists of a 32-basepair deletion in the open reading frame. It effectively results in functional CCR5 deficiency by absence of CCR5 membrane expression.[35] In the Caucasian population approximately 15% is heterozygous for the CCR5Δ32 allele and 1-2% homozygous.[36] The CCR5Δ32 effectively results in functional CCR5 deficiency by absence of CCR5 membrane expression in homozygotes and reduced membrane expression in heterozygotes.[35]

CCR5 is particularly interesting given the availability of an approved pharmacological antagonist, providing opportunities for intervention, and because of the existence of the aforementioned genetic variant CCR5Δ32 leading to CCR5 deficiency. The latter allows the study of "knock-down" of the human CCR5 gene on chronic inflammatory diseases like atherosclerosis in renal patients. Data on HIV epidemiology demonstrate that genetic deficiency of the CCR5 is associated with resistance to HIV infection, as CCR5 modulates virus entry.[37, 38] In line with a functional, protective effect against HIV infection, association studies showed that

CCR5Δ32 is associated with better outcome in patients with a high risk for atherosclerotic cardiovascular disease and renal transplant recipients,[39-42] albeit not invariably so.[43-45] Modulation of the inflammatory response was suggested to be involved in the effect of CCR5Δ32 on prognosis in cardio-renal conditions. In animal studies using mouse models the role of both pharmacological CCR5 receptor antagonism and genetic deletion of CCR5 in the atherosclerotic, inflammatory response has been verified.[46-55] However, owing to differences in atherosclerotic disease between human and mouse models care must be taken to in extrapolating these results to the clinic. Studies in human cells and tissue are critical in substantiating the role of CCR5 in disease development. Different human studies show that CCR5 is present in human atherosclerotic plaque.[31, 56] The mechanism by which CCR5 and CCR5 deficiency contribute to chronic inflammation and atherosclerosis are believed to be due to its effects on immune cell migration and response.[12, 57] Notably, in mice CCR5 deficiency was associated with a reduction in Th1-type immune response.[46, 49] Besides this, in animal models CCR5 deficiency modulates monocyte recruitment in atherosclerotic lesions and is associated with improved plaque stability.[11, 12, 48]

Taken together, CCR5 is a plausible candidate for modulation of the process of atherosclerosis, in particular atherosclerosis in the context of a chronic pro-inflammatory state. The studies in this thesis therefore focus on CCR5 as a possible target for intervention in the chronic inflammatory process of atherosclerotic disease in ESRD and type 2 diabetes. To this purpose, we studied the epidemiological and functional consequences of the CCR5Δ32 polymorphism in ERSD and type 2 diabetes.

Outline of the thesis

In chapter 2 we tested the hypothesis that CCR5Δ32 is associated with mortality in patients with type 2 diabetes. To this purpose we investigated whether the presence or absence of CCR5Δ32 is associated with overall and/or cardiovascular mortality in a longitudinal follow-up cohort of type 2 diabetic patients. Unfortunately, the possible interference with the inflammatory state could not be tested as data on inflammation were not available.

An elevated serum level of C-reactive protein (CRP) is an established marker of systemic inflammation.[58, 59] In dialysis patients CRP has been strongly associated with overall and cardiovascular mortality, supporting the impact of inflammation in particular in this high-risk group.[17, 60-63] In this process the CCR5 receptor might well contribute to atherogenesis through the binding of its ligands, which in turn mediate the recruitment of inflammatory cells to the endothelium. The presence of a dysfunctional receptor could be a rate-limiting factor in the increased mortality associated with systemic inflammation in dialysis patients. In chapter 3 we therefore tested the hypothesis that the CCR5Δ32 genotype might alter the previously observed association of elevated CRP with mortality in ESRD. Thus, we investigated whether the CCR5Δ32 genetic variant modifies the effect of CRP on mortality in a Dutch dialysis cohort (NECOSAD). For independent confirmation we analysed the corresponding associations in a Swedish cohort of ESRD patients.

Of the vast family of cytokines, TNF-α, IL-6 and IL-10 seem to play a major role in the development of Th1/Th2 imbalance, leading to increased (cardiovascular) complications and worse outcome in patients with ESRD.[64] In atherosclerosis in mice, CCR5 deficiency is associated with a more pronounced Th2 type immune response and less TNF-α and INF-γ production hereby counteracting the Th1 directed Th1/Th2 disequilibrium of atherosclerotic inflammation.[46, 48, 49, 53] In chapter 4 we hypothesize that the previously observed protection from CRP related (cardiovascular) mortality in CCR5Δ32 carriers (chapter 3) is (in part) due to blunting of the pro-inflammatory immune response in carriers of the deletion. We tested this in a cohort of incident dialysis patients in who levels of CRP, TNF-α, IL-6 and IL-10 were assessed and related to their CCR5Δ32 genotype. In chapter 5 this possible explanation for the protection by the CCR5Δ32 genotype due to a more Th2 type directed immune response was further tested by studying differences in cytokine level after stimulation of peripheral mononuclear cells (PBMCs) and by studying the distribution of Th1, Th2 and Th17 directed circulating CD4+ and CD8+ T cells, based on their intracellular cytokine profile and regulatory T cells after stimulation in patients with ESRD with and without the CCR5Δ32 genotype.

Development of novel pharmacological approaches followed by randomized clinical trials is expensive and time consuming, providing an immense obstacle to the development and introduction of innovative approaches in patient care.[65] Therefore, alternative strategies are urgently needed to facilitate the multi-faceted process from drug development to introduction in clinical practice. Observational studies using genetic variants might provide such a strategy. Data obtained through genetic association studies could be considered a type of natural, lifelong, clinical trial, with genetically different groups being randomized at conception, hereby limiting confounding. This approach is known as Mendelian randomization.[66-68] We found that CCR5Δ32, leading to CCR5 deficiency was associated with protection against mortality in patients with ESRD with elevated CRP as a sign of systemic inflammation.[69] These data suggest that intervention targeting the CCR5 may have the potential to improve prognosis in ESRD.[70] In line with the above, genetic association data on long term outcome in patients with CCR5Δ32 versus those with the wild-type genotype can be considered as a virtual long term randomized intervention study on pharmacological blockade of the CCR5 receptor, thus providing a rapid and cheap simulation set-up for a real-life clinical trial. In chapter 6 we use this concept to estimate the potential cost-effectiveness of CCR5Δ32 screening and pharmacological CCR5 blockade in patients with ESRD, from the perspective of the Dutch health-care system.

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Chapter 2

CCR5Δ32 genotype is associated with outcome in type 2 diabetes mellitus

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Abstract

Aim of this study was to test whether the genetic variant CCR5Δ32 in the CC-chemokine receptor 5, which is known to lead to CCR5 deficiency, is associated with mortality in type 2 diabetes patients.

We examined the effect of presence or absence of the CCR5Δ32 on overall and cardiovascular mortality risk in the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC) cohort, a type 2 diabetes patient cohort.

We studied 756 patients with a mean duration of follow-up of 5.4 (± 1.4) years. 194 patients died during follow up of which 83 were cardiovascular deaths. 144 subjects (19%) carried the CCR5Δ32 deletion. CCR5Δ32 carriers had an adjusted hazard ratio of 0.62 (95%CI: 0.40-0.96; p=0.03) for all-cause mortality and 0.63 (95%CI: 0.33-1.19; p=0.16) for cardiovascular mortality.

The presence of CCR5Δ32 is associated with better survival in type 2 diabetes patients. These data suggest that it is worthwhile to explore the protective potential of pharmacological blockade of CCR5 in type 2 diabetic patients.

Introduction

Chemokines and their receptors have a central role in leucocyte trafficking, and are involved in the pathophysiology of various inflammatory disorders.[1, 2] Genetic variability in the chemokine cascades could therefore potentially modify inflammatory processes. For the CC-chemokine receptor 5 (CCR5) several polymorphisms have been described. Among these, the CCR5Δ32 genetic variant, consisting of a 32-basepair deletion in the open reading frame effectively results in functional CCR5 deficiency by absence of CCR5 membrane expression. Heterozygous subjects express a lower amount of functional receptors compared to wild-type homozygotes.[3] The pathophysiological significance of the CCR5Δ32 genetic variant is demonstrated by its association with resistance to HIV infection, where CCR5 modulates virus entry.[4, 5]

CCR5 is expressed on T cells, macrophages, smooth muscle cells and endothelial cells.[6, 7] These cells are involved in the chronic inflammatory state present in insulin resistance, type 2 diabetes, atherosclerosis and uremia.[2, 8-10] In line with its protective effect in HIV, CCR5Δ32 has also been shown to be associated with better outcome in patients with a high risk for atherosclerotic cardiovascular disease, dialysis patients and renal transplant recipients, probably by modulation of the inflammatory response in these conditions.[11-15]

Type 2 diabetes is characterized by a particularly elevated overall and cardiovascular mortality, attributed at least partly to a generalized inflammatory condition.[8, 16] This elicits the hypothesis that CCR5Δ32 could be associated with mortality risk in type 2 diabetes as well. To test this hypothesis, in the current study we investigated whether the presence or absence of CCR5Δ32 is associated with overall and/or cardiovascular mortality in a longitudinal follow-up cohort of type 2 diabetic patients.

Patients and Methods

Patients

This study is part of the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC). In this project, general practitioners (GP) receive support by diabetic specialists for the implementation of the Dutch national guidelines in care of type 2 diabetic patients. Patients were recruited from the eastern part of the Netherlands. In a part of this project all patients with type 2 diabetes, exclusively treated by their GP are followed annually. These patients (n=1149) are part of the present study. Eligibility criteria were: type 2 diabetes, as defined by the national guidelines of the Dutch college of general practitioners (based on the 1997 American Diabetes Association

criteria) treated by a general practitioner. All patients gave informed consent before being included. The study was approved by the local medical ethics committee. Patients were included between January 1998 and December 1999. Details were described previously.[17] For the current analyses data were used from patients who gave permission for DNA analyses (n=798). Patients were followed until date of death or date of censoring, i.e. withdrawal from the study or end of the follow-up period (December 2004).

Demographic and clinical data

The following data were collected: age, gender, smoking habit, medication use, systolic and diastolic standing, office blood pressure after 5 minute rest (Tycos sphygmomanometer; Welch Allyn B.V., Delft, the Netherlands), medical history and co-morbidity, body mass index (BMI) and diabetes duration. A blood sample and a urine sample were obtained. Serum lipids, serum creatinine and urine albumine/kreatinine ratio were determined by routine assays (Roche/ Hitachi modular analyzer; Roche diagnostics, Laval, QC, Canada). HbA1C was determined by high performance liquid chromatography (Primus CLC-385; Primus Corp., Kansas City, MO, USA). Creatinine clerance was calculated using the MDRD formula.[18] Patients with a history of angina pectoris, myocardial infarction, heart failure, stroke or claudication at time of inclusion were defined as having cardiovascular disease. Dates of death were determined by reviewing patient records or were reported by the general practitioner and were checked at the Central Bureau of Statistics. The cause of death was obtained by linking the number of the death certificate to the primary cause of death as coded by a physician from the Central Bureau of Statistics. Cardiovascular causes of death were coded according to the International Classification of Diseases (ICD), 9th revision. Death due to ischemic coronary heart disease, heart failure, and cerebrovascular disease were coded as cardiovascular death.

DNA preparation and Polymerase Chain Reaction analysis

The CCR5 gene is located on chromosome 3p21. In the assay, genotypes were determined by discrimination during the polymerase chain reaction (PCR) with two allele-specific probes (PE Biosystems, Foster city, CA, USA). Each assay requires two unlabeled primers (Life Technologies, Foster city, CA, USA). The PCR was accomplished by using Taqman universal master mix (PE Biosystems, Foster city, CA, USA). A detailed description was published previously.[19] Patients were divided in 2 groups according to their CCR5 genotype namely those homozygous for the major allele (non-carriers) and those with 1 or 2 deletion alleles (carriers). Patients with one or two deletion alleles were grouped together, as it has been demonstrated that presence of one minor allele is sufficient to compromise CCR5 function.[3] Moreover, the number of individuals homozygous for the minor allele was too low to provide adequate statistical power to analyze as a separate group.

Statistics

Hardy-Weinberg equilibrium was calculated using the gene-counting method. Differences between groups were tested with the chi-square test for dichotomous and categorical variables and one-way ANOVA for continuous variables. Survival of overall and cardiovascular mortality was investigated using univariate and multivariate Cox's propotional-hazard analyses. A primary multivariate analysis included age and sex as possible confounders. In further multivariate analyses, additional adjustment was performed for variables with significant difference between the two genotype groups. Finally, additional adjustment was performed for variables with more than 10% difference between the two groups. Cumulative hazards were calculated to display the survival model graphically. All statistical analyses were performed with SPSS statistical software (version 14.0; SPSS, Chicago, IL, USA). A p-value of 0.05 was assumed to indicate statistical significance for all analyses.

Results

A total of 798 patients were included. In 42 patients (5.3%) the CCR5 genotype could not be determined. These patients showed similar baseline characteristics to the genotyped patients (data not shown). Further statistical analyses were performed on the 756 patients who were genotyped for CCR5.

The CCR5 ins32 (+)/del32 (Δ) genotype was distributed as follows: 613 +/+ (81.1%), 137 +/Δ (18.1%) and 6 Δ/Δ (0.8%). The genotype frequency did not deviate significantly from Hardy-Weinberg equilibrium (P=0.58). As stated in the methods section we combined carriers of the CCR5Δ32 genetic variant into a single carrier genotype group of 143 individuals (18.9%).

Table 1 lists the baseline characteristics of the population stratified by CCR5 genotype. The patient characteristics for the different genotype groups were largely similar, except systolic and diastolic blood pressure and HDL cholesterol level. Carriers of the Δ32 polymorphism had a higher systolic and diastolic blood pressure and a higher HDL cholesterol level. Also the use of lipid lowering drugs was different between both groups, with less use of lipid lowering drugs in carriers.

The mean follow-up duration was 5.4 ± 1.4 with a maximum of 6.8 years. In 46 (38 (6.2%) non-carriers and 8 (5.6%) carriers) of the 756 patients no follow-up data were available.

Table 1: Baseline characteristics of the ZODIAC cohort

	CCR5 +/+ (n=613)	CCR5 +/Δ and Δ/Δ (n=143)	p
Male sex (%)	42.4	38.5	0.39
Age (years)	68 ± 11	68 ± 10	0.44
Smoking (%)			
Current	18.8	14.0	0.41
Never	51.3	54.4	
Previously	29.8	31.6	
CVD (%)	36.2	28.7	0.10
DM duration (years)	7.2 ± 8.0	6.2 ± 6.1	0.17
SBP (mmHg)	153 ± 25	159 ± 27	0.01
DBP (mmHg)	84 ± 12	86 ± 11	0.04
BMI (kg/m²)	28.8	28.7	0.80
TC (mmol/l)	5.6 ± 1.1	5.8 ± 1.1	0.10
HDL-c (mmol/l)	1.16 ± 0.33	1.23 ± 0.34	0.03
HbA1C (mmol/l)	7.4 ± 1.2	7.3 ± 1.3	0.19
eGFR (ml/min)	75.9 ± 17.7	76.2 ± 16.8	0.89
Alb/creat ratio > 2.5 males, > 3.5 females	36.8	40.2	0.48
ADT (%)			
No medication (only diet)	12.2	12.4	
Only oral drugs	73.1	69.9	0.76
Only insulin	12.8	16.2	
Insulin and oral drugs	1.9	1.5	
AHT (%)	43.4	39.7	0.43
LLD (%)	11.3	5.1	0.03
Aspirin (%)	12.0	9.6	0.43

CVD, history of cardiovascular disease; ADT, anti-diabetic treatment; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; TC, total cholesterol; HDL-c, HDL-cholesterol; eGFR, estimated GFR by MDRD formula; AHT, anti-hypertensive treatment; LLD, lipid-lowering drugs. Data are presented as percentage or mean (SD).

A total of 194 (25.7%) patients died during the follow-up period at an average of 4.8% per year: 167 (27.2%) in the non-carrier group and 27 (18.8%) in the carrier group. None of the six patients homozygous for the deletion allele died during follow-up. From the total number of deaths 83 (42.7%) were of cardiovascular cause (72 (11.7%) in the non-carrier and 11 (7.7%) in the carrier group). In 44 patients the cause of death was classified as due to malignancy, in 15 patients cause of death was classified as due to respiratory causes, in 5 patients cause of death was classified as trauma, and in 47 patients death was due to other causes. These causes of death were distributed equally between the two genotype groups.

Table 2: Hazard ratios, 95%CI and p-values for all-cause and cardiovascular mortality by CCR5Δ32 genotype

	Crude overall mortality	p-value	Adjusted overall mortality	p-value
CCR5 +/+	1		1	
CCR5 +/Δ and Δ/Δ	0.64 (0.41-0.98)	0.04	0.62 (0.40-0.96)	0.03

	Crude cardiovascular mortality	p-value	Adjusted cardiovascular mortality	p-value
CCR5 +/+	1		1	
CCR5 +/Δ and Δ/Δ	0.64 (0.34-1.21)	0.17	0.63 (0.33-1.19)	0.16

Non-carriers (CCR5 +/+) were used as reference in Cox regression analysis. In the crude model no further adjustments were made. In the adjusted model, age and sex were included as potential confounders.

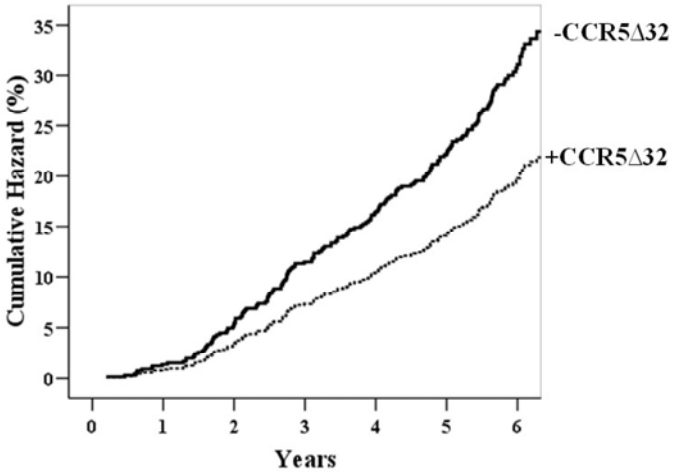

Figure 1: Cumulative hazard (%) for all-cause mortality in DM II patients stratified by presence of the CCR5Δ32 minor allele. Non-carriers (-CCR5Δ32) show a higher (p=0.038) hazard for mortality compared to carriers (+CCR5Δ32).

In multivariate Cox regression analyses (adjusted for age and sex), the hazard ratios of CCR5Δ32 carriers compared to non-carriers were 0.62 (95% CI: 0.40-0.96, p=0.03) for all-cause mortality and 0.63 (95% CI: 0.33-1.19, p=0.16) for cardiovascular mortality (table 2). For mortality due to ischemic heart disease (n=31) alone, the adjusted hazard ratio was 0.29 (95% CI: 0.07-1.21; p=0.09). The adjusted hazard ratio for non-cardiovascular mortality was 0.61 (95% CI: 0.34-1.10, p=0.10). Further adjustment for factors that were significantly different at baseline between the two genotype groups (blood pressure, HDL cholesterol and lipid lowering medication) did not alter the results. In un additional multivariate model, adjustment for factors that were not significantly different but had a more than 10% difference between the two genotype groups at baseline (CVD, diabetic duration and anti-diabetic treatment) did not materially affect the results (data not shown). Figure 1 illustrates the survival model for all-cause mortality depending on CCR5Δ32 genotype.

Discussion

In this longitudinal follow-up in type 2 diabetes patients the presence of the CCR5Δ32, leading to CCR5 deficiency, was associated with lower all-cause mortality. Hazard ratios for cardiovascular and non-cardiovascular mortality and especially mortality due to ischemic heart disease were also lower in carriers of the CCR5Δ32 variant but these differences did not reach statistical significance. These data are the first to demonstrate the association of CCR5Δ32 with mortality in patients with diabetes type 2.

These data are in line with the impact of the CCR5Δ32 in several other populations. Data in HIV infection, where CCR5 modulates virus entry, provided the first evidence for pathophysiological impact of the CCR5 deficiency that is conferred by CCR5Δ32 as carriers showed resistance against HIV infection.[5] Causality was supported by a recent case report on a patient with acute myeloid leukaemia and HIV infection, who remained without viral rebound after transplantation with stem cells from a donor homozygous for CCR5Δ32.[4] Case-control studies in cardiovascular disease demonstrated that CCR5Δ32 is associated with a reduced incidence of myocardial infarction at younger age in men and with protection against coronary heart disease.[12, 14] In a nested case-control study within the Nurses' Health Study a possible association was found between reduced incidence of early onset coronary heart disease in women.[13] Indeed, in animal studies, it has been suggested that CCR5 and its ligands play a role in the pathogenesis and progression of vascular disease.[20-22] From the function of CCR5 it would be logical to assume that CCR5Δ32 modulates inflammatory responses. In line with this assumption, CCR5Δ32 is associated with protection against rheumatoid arthritis and with better

outcome in renal transplantation, a condition characterized by persistent inflammation.[11, 23] Recently we demonstrated gene-environment interaction between CCR5Δ32 and inflammatory status in two independent cohorts of dialysis patients, where CCR5Δ32 abolishes the well-established association between elevated CRP and mortality.[15] Taken together these studies suggest that CCR5Δ32 modulates outcome in various inflammatory-driven disease processes. Our current data extend these findings to type 2 diabetes.

Our study was not designed to address the mechanisms underlying the impact of CCR5Δ32 on mortality, but several inferences can be made. Most likely a dysfunctional CCR5 could be related to lower over-all mortality by modulating inflammatory responses. Interestingly, a Polish study reported over expression of CCR5 on circulating blood mononuclear cells in type 2 diabetic patients compared to healthy controls.[24] In addition, high plasma levels of the CCR5 ligand CCL5 were associated with increased cardiac mortality.[25] Another study by Boger et al. suggested that up regulation of CCR5 could lead to accelerated atherosclerosis in type 2 diabetes mellitus patients on hemodialysis.[26] Also other CCR5 polymorphisms have been implicated in the development of diabetic complications.[27, 28] Together these findings support involvement of CCR5-mediated inflammatory processes in the outcome of diabetes. Thus, the CCR5 deficiency resulting from the CCR5Δ32 variant could explain why carriers had a better survival in our study.

What could be the implications of our findings? First, they could contribute to risk stratification. Moreover, they support the rationale for pharmacological blockade of the CCR5 as a preventive strategy. This idea is supported by animal data showing that the CCR5 antagonist Met-RANTES reduced progression of atherosclerosis in hypercholesterolemic mice and with reduced neo-intimal plaque area and macrophage infiltration in apoE deficient mice.[21, 29] Finally, treatment with TAK-799, a CCR5 chemokine receptor antagonist, reduced lesion development in a collar-induced carotid artery atherosclerosis model.[30] In humans, pharmacological blockade of the CCR5 is also feasible, as recently this strategy has been introduced for treatment of HIV infection, but so far no experience is available in other conditions.[31]

Our study has several limitations. We excluded a number of patients for which CCR5 genotype was not determined that could potentially introduce a selection-bias. However, it is highly unlikely that this sporadic technical failure would distribute unequally among patients, as patient characteristics were similar in genotyped versus non-genotyped subjects. Another limitation is that causes of death could have been misdocumented and hereby may have biased the result concerning cardiovascular and non-cardiovascular mortality. However, this could not have influenced our main outcome, ie. all-

cause mortality. The incidence of mortality in our study population is comparable to that reported in literature, suggesting that in terms of mortality, our study population resembles a ordinarily type 2 diabetes population.[32] Population stratification is a form of confounding that may occur in genetic association studies when a distinct population comprises subgroups with different genetic background. Unfortunately, data on ethnicity were not recorded. However in the region of the Netherlands were the study was performed the vast majority of inhabitants is of Caucasian origin. Besides this the genotype did not deviate from Hardy Weinberg equilibrium. So, this form of confounding is not likely to play an important role. To overcome bias through selection and population stratification replication of our findings in an independent population would have been helpful. Whereas this is a single center study, and no formal replication is provided, our data are in line with those in other populations, thus supporting its credibility.

In genetic association studies adjustment for other factors than age and sex could potentially introduce interference in the causal pathway and thereby bias through overadjustment.[33] For this reason we reported unadjusted hazard ratios and hazard ratios adjusted for age and sex in the manuscript. Even adjustment for the factors that were at baseline significantly different or showed a more than 10% difference between the 2 genotype groups did not materially affect our conclusions. Moreover, as CCR5Δ32 carriers survived longer despite higher blood pressure it could be hypothesized that carriers are more resistant to adverse events related to elevated blood pressure. Similarly, less lipid lowering treatment in CCR5Δ32 carriers apparently did not adversely affect their survival. Finally, we only studied a single polymorphism. The observed effect does not necessarily causally implicate this particular polymorphism, but could be due to another variant in linkage disequilibrium with the studied deletion. This is a point that deserves further investigation. However, our efforts as reported in the present study were not toward in-depth characterization of the gene locus, but rather to investigate whether the reported impact in the literature of CCR5Δ32, leading to CCR5 deficiency, was also present in a diabetic population.

CCR5 deficiency due to the presence of the CCR5Δ32 genotype, is associated with improved survival in type 2 diabetes. These data are in line with previous data and support the pathophysiological impact of the CCR5. They suggest that pharmacological blockade of the CCR5, which has recently become feasible in humans, could have the potential to improve prognosis in type 2 diabetes.

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Chapter 3

CCR5 deletion protects against inflammation-associated mortality in dialysis patients

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Abstract

A genetic polymorphism of the CC-chemokine receptor 5 (CCR5Δ32) leads to CCR5 deficiency. We hypothesized that CCR5Δ32 modulates inflammation driven mortality in end stage renal disease (ESRD) patients and therefore studied the interaction between CCR5 genotype and CRP levels.

We included 603 patients from the NEtherlands COoperative Study on the Adequacy of Dialysis (NECOSAD) cohort, a multicenter prospective follow-up study in incident dialysis patients. In 413 patients (68%) the CCR5 genotype and hsCRP levels were available. Patients were followed for a median of 3.75 years.

A total of 170 patients died during 5 years of follow-up (87 cardiovascular). By combining the presence or absence of the CCR5Δ32 polymorphism with hsCRP levels effect modification was studied. This showed that compared to the reference group, ie. patients with the wild type genotype and hsCRP≤10mg/l (n=225), patients carrying the deletion allele with hsCRP≤10mg/l (n=55) had the same survival (HR: 0.90 (95%CI: 0.54-1.50; p=0.69). Patients carrying the wild type genotype with hsCRP>10mg/l (n=108) had an increased mortality (HR: 1.82 (95%CI: 1.29-2.58; p<0.01). However, in carriers of the deletion allele with hsCRP>10mg/l (n=25) survival was the same as in the reference group (HR: 1.39 (95%CI: 0.73-2.62; p=0.32)). This effect was even more pronounced for cardiovascular mortality. This finding was replicated in an independent Swedish cohort of 302 ESRD patients.

The CCR5Δ32 polymorphism attenuates the adverse effects of an inflammatory state on overall and cardiovascular mortality in ESRD.

Introduction

Cardiovascular disease (CVD) is a prominent cause of mortality in end-stage renal disease (ESRD) patients.[1] A persistent inflammatory state has been recognized a risk factor in this respect. An established marker of systemic inflammation is an elevated serum level of C-reactive protein (CRP).[2, 3] Although CRP has been strongly associated with overall and cardiovascular mortality in dialysis patients, recent evidence suggests that CRP itself does not have an atherogenic potential.[4-9]

The inflammatory process in atherosclerosis is characterized by infiltration of monocytes and T-lymphocytes in the vascular wall in response to chemokines. Different studies suggest that the chemokines CCL5/RANTES, CCL3/macrophage inflammatory protein (MIP)-1α and CCL4/MIP-1β and their receptor CC-chemokine receptor 5 (CCR5) play a role in the pathogenesis of atherosclerosis.[10-14] CCR5 is expressed on the principal cell types implicated in atherogenesis.[15-18]

In states of inflammation the CCR5 receptor could contribute to atherogenesis through the binding of its ligands, which in turn mediate the recruitment of inflammatory cells to the endothelium. Interestingly, patients with a dysfunctional CCR5 due to the gene polymorphism CCR5 deletion 32 (CCR5Δ32), a 32-basepair deletion in the open reading frame leading to premature termination of the protein and sequestration in the endoplasmic reticulum, have an improved prognosis in atherosclerotic diseases.[19-23] Thus, CCR5Δ32 could be a rate-limiting factor in the increased mortality rate associated with systemic inflammation.

We therefore hypothesized that the CCR5Δ32 polymorphism might alter the previously observed association of elevated CRP with mortality in ESRD. To test this hypothesis we investigated whether the CCR5Δ32 polymorphism modifies the effect of CRP on mortality in a Dutch dialysis cohort (NECOSAD). For independent confirmation, we analysed the corresponding associations in a Swedish cohort of ESRD patients.

Methods

Patients

This study is part of the NEtherlands COoperative Study on the Adequacy of Dialysis (NECOSAD). This is a multicenter prospective follow-up study in which new ESRD patients from 38 Dutch dialysis centers are included at start of chronic dialysis treatment. All patients gave informed consent and all local medical ethics committees gave their approval.

Eligibility criteria were: 18 years and older and no previous renal replacement therapy. For the current analyses, data were used from patients included between July 1998 and December 2001 in 23 centers that approved of DNA analysis and had a follow-up of at least 3 months. An additional criterion was that the response rate was more than 50% for DNA analyses of patients in these centers.

Demographic and clinical data

The collected data included: age, gender, smoking, primary kidney disease, systolic and diastolic blood pressure, co-morbidity, dialysis modality and medication use. Blood and 24-hour urine samples were obtained at 3 months after start of dialysis. Plasma hemoglobin, creatinine, urea, albumin and cholesterol levels were determined. High sensitivity CRP (hsCRP) was measured by means of particle-enhanced immunonephelometry using a standard CardioPhase hsCRP for BNII (Dade Behring Holding GmbH, Liederbach, Germany. Detection limit 0.1 mg/l, precision 0.1 mg/l).[6] In addition, blood was collected for DNA analysis. Urea and creatinine were also analyzed in the urine sample. Renal function, expressed as glomerular filtration rate (GFR), was calculated as the mean of creatinine and urea clearance, corrected for body surface area (mL/min/1.73m²). Patients were followed at 3 and 6 months after start of dialysis and thereafter every 6 months until date of death or date of censoring, i.e. transfer to a non-participating dialysis center, withdrawal from the study or end of the follow-up period in June 2007. Patients receiving a kidney transplant were not censored.

Clinical definitions

Primary kidney disease and causes of death were classified according to the codes of the European Renal Association – European Dialysis and Transplantation Association (ERA-EDTA).[24] The following codes were designated as cardiovascular mortality: myocardial ischemia and infarction; cardiac failure/ fluid overload/ pulmonary oedema; cardiac arrest, cause unknown; cerebro-vascular accident; haemorrhage from ruptured vascular aneurysm; mesenteric infarction; hyperkalaemia; hypokalaemia; cause of death uncertain/ unknown. The patients were grouped in 4 classes of primary kidney diseases: glomerulonephritis, diabetes mellitus, renal vascular disease and other kidney diseases. Other kidney diseases consisted of patients with interstitial nephritis, polycystic kidney diseases, other multi-system diseases and unknown diseases.

Patients with a history of angina pectoris, myocardial infarction, heart failure, ischemic stroke or claudication at time of inclusion were defined as having cardiovascular disease. Smoking habits are defined as never, current or former smoker.

Systemic inflammation was defined as hsCRP concentration above 10mg/l. This cut-off point was previously used to divide patients in a group with or without a chronic inflammatory state.[6] In ESRD patients this cut-off point for CRP level has been shown to be the best with regard to the prediction of survival.[25]

CCR5 polymorphism

The CCR5 gene is located on chromosome 3p21. The genotypes were determined with a PCR-based allelic discrimination assay using primers (Life Technologies) and allele-specific probes (PE Biosystems) as described previously.[26]

Patients were divided in 2 groups based on their CCR5Δ32 genotype: those homozygous for the major allele (functional receptor) and those with 1 or 2 deletion alleles (dysfunctional receptor). Patients homo- or heterozygous for the deletion-allele were clustered since the presence of one minor allele has already been associated with reduced receptor function [27].

Replication

For independent replication of the NECOSAD study results data from a Swedish cohort of ESRD patients were analysed. This is a prospective follow-up study in patients with ESRD close to the start of renal replacement therapy done in Sweden. Only patients older than 18 years and younger than 70 years were included. Patients who were hospitalized with clinical signs of infection and/or acute vasculitis at time of admission were not included. Patients were examined and blood was collected close to start of dialysis treatment. A detailed description was published previously.[28] Genotype analyses were performed by PCR amplification followed by size separation of the resulting fragments on a 3% agarose gel and visualization by ethiduim bromide staining. The resulting fragments were 241 and 209 bp for the ins and the del allele, respectively. Patients were followed until date of death or date of censoring, i.e. end of the follow-up period (March 2007). Definitions for cardiovascular disease, cardiovascular death, systemic inflammation were the same as described above for the NECOSAD cohort.

Statistics

Hardy-Weinberg equilibrium was calculated using the gene-counting method. Differences

between groups were tested with the chi-square test for dichotomous and categorical variables and one-way ANOVA for continuous variables. The main outcome measure was all-cause and cardiovascular mortality within five years of follow-up. The survival curves were determined with the Kaplan-Meier method. The log rank test was used to determine differences between survival curves. Unadjusted, adjusted (for gender, age at inclusion,

cardiovascular disease, diabetes mellitus and dialysis modality) hazard ratios (HRs) for all-cause, cardiovascular and non-cardiovascular mortality were calculated by Cox's proportional-hazard analysis.

In order to study the modification of the effect of hsCRP on mortality by the CCR5 genotype according to Rothmans approach of studying effect modification (i.e. additive interaction) [29] between hsCRP and CCR5 genotype, a new variable with four categories was defined: CCR5 ins/ins with low hsCRP (≤10mg/l), CCR5 ins/ins with high hsCRP (>10mg/l), CCR5Δ32 with low hsCRP (≤10mg/l) and CCR5Δ32 with high hsCRP level (>10mg/l).

This association was also examined for hsCRP level as a continuous variable by studying its effect on mortality within the two separate CCR5 genotype groups. Because of the skewed distribution, hsCRP data were first log-transformed.

Finally, to increase the number of patients in the different categories and hereby power, the 2 cohorts were combined. For the combined cohort Cox's proportional-hazard analysis was performed also.

All statistical analyses were performed with SPSS statistical software (version 14.0; SPSS, Chicago, IL).

Results

A total of 603 patients were included in the present analysis. In 413 patients (68%) the CCR5 genotype and hsCRP levels were available. Compared to the 190 patients without hsCRP and/or CCR5 data, these patients were slightly older (59.7 vs 57.1 (p=0.05)) and included more HD patients (67% vs. 52% (P<0.01)). Comorbidities such as diabetes mellitus did not differ in both patients groups (35% vs. 30% (p=1.19), and 25% vs. 20% (p=0.11), respectively). No differences were found in serum albumin (35.7 vs. 36.3 g/l (p=0.25)). However, a better survival was observed for the 413 patients with both hsCRP and CCR5 data with a median of 1370 days (3.75 years) (710-1826 days) of follow-up, and a mortality rate of 122 per 1000 person years within 5 years of follow-up, compared to a median of 1006 days of follow-up and a mortality rate of 147 per 1000 person years within 5 years of follow up for the 190 patients without hsCRP and/or CCR5 data. Further statistical analyses were performed on the 413 patients.

The CCR5 ins32/del32 polymorphism was distributed as follows: ins/ins: 333 (80.6%); ins/del: 73 (17.7%) and del/del: 7 (1.7%). The genotype distribution did not deviate significantly from Hardy-Weinberg equilibrium (p=0.21).

Baseline characteristics are shown in the first column of Table 1. The patient characteristics for the different genotype groups were similar at the start of dialysis, except antihypertensive medication use. Patients homo- or heterozygous for the deletion allele used more antihypertensive medications (p=0.01). The median CRP levels in the two genotype groups were as follows: in the CCR5 ins/ins group: 4.7 mg/l (1.8-13.4) and in the CCR5 del group: 6.9 mg/l (2.4-14.1) (p=0.22).

Table 1: Baseline characteristics of the NECOSAD and Swedish cohorts.

	NECOSAD N=413	**Swedish cohort** N=302	
Gender: male	253 (61.3)	185 (61.3)	
Age (year)	62 (50-71)	55 (44-64)	
Caucasian	379 (91.8)		
Hemodialysis	277 (67.1)	142 (47.0)	
Peritoneal dialysis	136 (32.9)	160 (53.0)	
Primary kidney disease			
Diabetes mellitus	75 (18.2)	88 (29.1)	Diabetic nephropathy
Glomerulonephritis	48 (11.6)	84 (27.8)	Chronic glomerulonephritis
Renal vascular	76 (18.4)	12 (4.0)	Nephrosclerosis
disease	214 (51.8)	35 (11.6)	Polycystic kidney disease
Other		83 (27.5)	Other
Cardiovascular disease	144 (34.9)	99 (32.8)	
Diabetes mellitus	105 (25.4)	88 (29.1)	
Smoking			
Never	120 (29.2)		
Former	194 (47.2)		
Current	97 (23.6)		
DBP (mmHg)	83 (12.8)	88 (13.2)	
SBP (mmHg)	150 (25.4)	151 (23.8)	
Antihypertensive medication	356 (86.2)		
Lipid lowering medication	121 (29.3)		
hsCRP (mg/l)	5.1 (1.9-	4.9 (2.0-14.0)	
hsCRP > 10 (mg/l)	13.7)	101 (33.4)	
	133 (32.2)		
Cholesterol (mmol/l)	5.0 (1.3)	5.3 (1.5)	
Albumin (g/l)	32.5 (6.9)	33.2 (6.1)	
Hemoglobin (g/dl)	11.0 (1.4)	10.4 (1.4)	
GFR (ml/min)	4.2 (3.1)	6.6 (2.3)	
Kt/V /week	2.3 (0.9)	-	
CCR5			
Ins/ins	333 (80.6)	246 (81.5)	
Ins/del32	73 (17.7)	51 (16.9)	
Del32/del32	7 (1.7)	5 (1.7)	

Data are presented as number (percentage), median (25, 75 percentile), mean (SD). DBP: diastolic blood pressure; SBP: systolic blood pressure; GFR: glomerular filtration rate.

Mortality and systemic inflammation

A total of 170 (87 (51 %) cardiovascular) patients died during follow-up of 5 years. The mortality rate in the group with hsCRP≤10mg/l was 91 per 1000 person years as opposed to 207 per 1000 person years in the group with hsCRP>10mg/l. Kaplan-Meier survival analysis showed a statistically significant difference in all-cause, cardiovascular and non-cardiovascular mortality for the two hsCRP level groups; those with hsCRP>10mg/l had the worst survival (log rank: p<0.01). These univariate findings were confirmed by Cox regression analysis (adjusted HR for patients with hsCRP≤10mg/l compared to hsCPR>10mg/l for all-cause mortality: 1.78 (95%CI: 1.31-2.42; p<0.01), for cardiovascular mortality: 1.70 (95%CI: 1.10-2.63; p=0.02) and for non-cardiovascular mortality: 1.87 (95%CI: 1.20-2.91; p<0.01)).

When analysed as a continuous variable the adjusted mortality risk per unit hsCRP increase was 1.31 (95%CI: 1.17-1.48; p<0.01) for all-cause mortality, 1.24 (95%CI: 1.04-1.46; p=0.01) for cardiovascular mortality and 1.41 (95%CI: 1.18-1.67; p<0.01) for non-cardiovascular mortality.

Mortality and systemic inflammation and CCR5 polymorphism interaction

Of the 170 patients who died within 5 years of FU, 140 patients were non-carriers of the CCR5Δ32 polymorphism (42% of all non-carriers), and 30 patients were carriers of the polymorphism (38% of carriers).

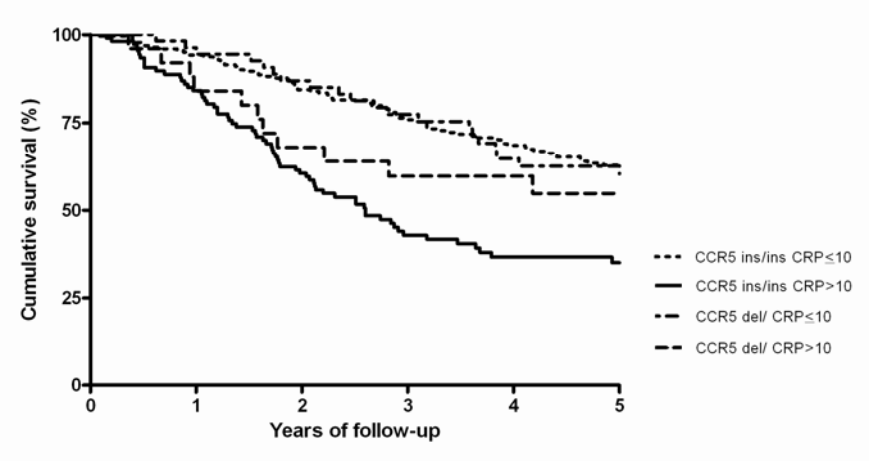

Figure 1: Kaplan-Meier overall survival curve for patients in the NECOSAD cohort according to CCR5 genotype combined with or without elevated hsCRP (mg/l) levels (log rank: p<0.001)

The Kaplan Meier survival curve (Figure 1) shows that patients with hsCRP>10mg/l and not carrying a deletion allele had the worst survival (log rank: p<0.01). In Table 2 the HRs for all-cause mortality are presented. In the NECOSAD cohort non-carriers of the deletion allele with hsCRP>10mg/l had an increased all-cause mortality risk compared to non-carriers with hsCRP≤10mg/l. Patients in the group with hsCRP>10mg/l, carrying the deletion allele showed a much less elevated HR for all-cause mortality. This effect was even more pronounced for cardiovascular mortality (Table 2). For non-cardiovascular mortality the protective effect of the deletion allele in patients with an elevated hsCRP was less pronounced (Table 2).

Table 2: Mortality rate per 1000 person years, hazard ratio (HR) (95%CI) and p-values for all-cause, cardiovascular and non-cardiovascular mortality in the NECOSAD cohort and hazard ratio (HR) (95%CI) and p-values for all-cause mortality in the Swedish cohort. Adjusted for gender, age at inclusion, cardiovascular disease, diabetes and dialysis modality.

NECOSAD	**CCR5 ins/ins & hsCRP≤10mg/l (n=225)**	**CCR5 ins/ins & hsCRP>10mg/l (n=108)**	**CCR5Δ32 & hsCRP≤10mg/l (n=55)**	**CCR5Δ32 & hsCRP>10mg/l (n=25)**
All-cause mortality				
Mortality rate	91	228	90	133
Crude HR	1	2.55 (1.83-3.56)	1.00 (0.60-1.65)	1.48 (0.79-2.79)
p		<0.01	0.99	0.22
Adjusted HR	1	1.82 (1.29-2.58)	0.90 (0.54-1.50)	1.39 (0.73-2.62)
p		<0.01	0.69	0.32
Cardiovascular mortality				
Mortality rate	47	112	52	60
Crude HR	1	2.50 (1.56-4.00)	1.11 (0.57-2.17)	1.30 (0.51-3.31)
p		<0.01	0.76	0.58
Adjusted HR	1	1.85 (1.13-3.01)	1.05 (0.53-2.07)	1.25 (0.49-3.17)
p		0.01	0.89	0.64
Non-cardiovascular mortality				
Mortality rate	44	116	38	72
Crude HR	1	2.60 (1.62-4.19)	0.88 (0.41-1.88)	1.67 (0.71-3.97)
p		<0.01	0.73	0.24
Adjusted HR	1	1.82 (1.12-2.97)	0.76 (0.35-1.65)	1.52 (0.64-3.63)
p		0.02	0.49	0.34
SWEDISH	**(n=167)**	**(n=79)**	**(n=34)**	**(n=22)**
All-cause mortality				
Crude HR	1	2.44 (1.50-3.99)	1.50 (0.72-3.15)	1.50 (0.66-3.40)
p		<0.01	0.28	0.33
Adjusted HR	1	1.67 (1.01-2.77)	1.95 (0.91-4.19)	0.94 (0.41-2.17)
p		0.05	0.09	0.89

Table 3: Hazard ratio (HR) (95%CI) and p-values for all-cause, cardiovascular and non-cardiovascular mortality per unit hsCRP increase in the NECOSAD cohort and hazard ratio (HR) (95%CI) and p-values for all-cause mortality per unit hsCRP increase in the Swedish cohort. Adjusted for gender, age at inclusion, cardiovascular disease, diabetes and dialysis modality.

NECOSAD	CCR5 ins/ins	CCR5Δ32
All-cause mortality		
Crude HR	1.52 (1.34-1.71)	1.31 (0.93-1.84)
P	<0.01	0.13
Adjusted HR	1.34 (1.18-1.52)	1.32 (0.89-1.97)
P	<0.01	0.17
Cardiovascular mortality		
Crude HR	1.47 (1.24-1.75)	1.05 (0.68-1.61)
p	<0.01	0.83
Adjusted HR	1.30 (1.08-1.55)	1.02 (0.61-1.68)
p	<0.01	0.95
Non-cardiovascular mortality		
Crude HR	1.56 (1.31-1.86)	1.79 (1.02-3.14)
p	<0.01	0.04
Adjusted HR	1.39 (1.16-1.66)	2.37 (1.16-4.86)
p	<0.01	0.02
SWEDISH		
All-cause mortality		
Crude HR	1.40 (1.17-1.66)	1.18 (0.82-1.69)
p	<0.01	0.36
Adjusted HR	1.18 (0.98-1.43)	0.73 (0.46-1.15)
p	0.08	0.17

In Table 3, the HRs for all-cause, cardiovascular and non-cardiovascular mortality for hsCRP level as a continuous variable for the 2 genotype groups are presented: hsCRP levels are associated with mortality in patients with the CCR5 ins/ins genotype and not in patients carrying a deletion allele, for cardiovascular mortality the results are more pronounced. For non-cardiovascular mortality in both CCR5 genotype groups there was a significant increase in mortality risk per unit hsCRP increase.

Limiting the analysis to patients with hsCRP level <50mg/l to exclude patients who could have had an acute infection showed the same HRs for overall and cardiovascular mortality. The HRs for non-cardiovascular mortality for the group with hsCRP>10mg/l, carrying the deletion allele resulted in a lower value (adjusted HR: 1.08 (95%CI: 0.38-3.04; p=0.89)).

Also, limiting the analysis to Caucasian patients resulted in comparable HRs (data not shown).

By taking the median hsCRP level as cut-off point instead of using a cut off hsCRP level of 10mg/l to divide patients into two groups (with or without a systemic inflammation respectively), yielded similar results. Also, extending the follow-up to more than 5 years did not significantly change the results; neither did further adjusting for primary kidney disease, smoking, blood pressure and medication use (data not shown).

Independent replication

The population used for confirmation consisted of 302 ESRD patients characterized for CCR5 genotype and hsCRP level. Baseline characteristics are given in the second column of Table 1. There were no differences in baseline characteristics between CCR5Δ32 carriers and non-carriers. The causes of primary kidney disease differed between the NECOSAD and the Swedish cohort. Also, compared to the NECOSAD study population, the Swedish cohort was by inclusion criteria significantly younger and a lower proportion of patients started on hemodialysis. Whereas mean diastolic blood pressure, cholesterol levels and GFR were higher, the hemoglobin level was lower. The median follow-up was 1457 days (3.99 years) (712-1826). The CCR5Δ32 genotype distribution did not deviate significantly from Hardy-Weinberg equilibrium (p=0.22) and the allele frequencies did not differ from the NECOSAD cohort (p=0.96). During 5 years of follow-up 80 patients died (57 cardiovascular): 64 (26%) patients without the CCR5Δ32 polymorphism and 16 (29%) of the patients carrying the CCR5Δ32 polymorphism. In accordance with the NECOSAD cohort, in the Swedish cohort non-carriers of the deletion allele with an elevated hsCRP level had an increased mortality rate (Table 2). Similarly, carriers of the deletion allele without and with an elevated hsCRP showed a lower effect estimate in survival. For cardiovascular mortality the HRs with CCR5 ins/ins & hsCRP≤10 as reference were as follows; CCR5 ins/ins & hsCRP>10: 2.55 (95%CI: 1.44-4.51; p<0.01), adjusted 1.69 (95%CI: 0.94-3.05; p=0.08); CCR5Δ32 & hsCRP≤10: 1.17 (95%CI: 0.44-3.06; p=0.76), adjusted 1.73 (95%CI: 0.64-4.68; p=0.28); CCR5Δ32 & hsCRP>10: 1.50 (95%CI: 0.57-3.95; p=0.41), adjusted 0.98 (95%CI: 0.37-2.61; p=0.96). For non-cardiovascular mortality there were no statistically significant differences between the four groups.

In Table 3 the HRs for all-cause mortality for hsCRP level as a continuous variable for the 2 genotype groups are presented. For cardiovascular mortality the unadjusted and adjusted risks were respectively 1.43 (95%CI: 1.17-1.76; p<0.01) and 1.24 (95%CI: 1.00-1.55; p=0.05) in the CCR5 ins/ins genotype group and respectively 1.20 (95%CI: 0.76-1.89; p=0.45) and 0.55 (95%CI: 0.27-1.11; p=0.09) in the CCR5Δ32 genotype group.

Table 4: Hazard ratio (HR) (95%CI) and p-values for all-cause, cardiovascular and non-cardiovascular mortality in the combined cohort. Adjusted for gender, age at inclusion, cardiovascular disease, diabetes and dialysis modality

NECOSAD + SWEDISH	CCR5 ins/ins & hsCRP≤10mg/l (n=392)	CCR5 ins/ins & hsCRP>10mg/l (n=187)	CCR5Δ32 & hsCRP≤10mg/l (n=89)	CCR5Δ32 & hsCRP>10mg/l (n=47)
All-cause mortality				
Crude HR	1	2.46 (1.87-3.25)	1.16 (0.77-1.76)	1.42 (0.86-2.34)
p		<0.01	0.49	0.17
Adjusted HR	1	1.73 (1.31-2.30)	1.09 (0.72-1.66)	1.23 (0.74-2.02)
p		<0.01	0.69	0.42
Cardiovascular mortality				
Crude HR	1	2.48 (1.72-3.56)	1.14 (0.66-1.98)	1.36 (0.70-2.65)
p		<0.01	0.63	0.37
Adjusted HR	1	1.76 (1.21-2.54)	1.16 (0.67-2.03)	1.15 (0.59-2.25)
p		<0.01	0.59	0.68
Non-cardiovascular mortality				
Crude HR	1	2.45 (1.60-3.74)	1.18 (0.63-2.23)	1.50 (0.71-3.18)
p		<0.01	0.61	0.29
Adjusted HR	1	1.70 (1.10-2.62)	1.03 (0.54-1.95)	1.31 (0.62-2.79)
p		0.02	0.94	0.48

Combining the Swedish cohort with the NECOSAD cohort gave comparable results (with smaller confidence intervals) as initially found in the NECOSAD population (Table 4).

Discussion

Our prospective study of Dutch incident dialysis patients suggest that mortality in ESRD associated with elevated serum hsCRP concentrations is modulated by the CCR5Δ32 polymorphism. Elevated hsCRP was significantly associated with decreased survival in patients who were homozygous for the major allele and thus had a functional receptor. Interestingly, even though elevated hsCRP conferred an increased hazard for mortality in carriers of the deletion allele, this was a third of the magnitude observed in non-carriers and not significant. For cardiovascular mortality this suggested protective effect of a deletion allele was even more pronounced. For all-cause mortality, this finding was replicated in an independent Swedish cohort of ESRD patients. HRs for cardiovascular mortality showed the same trend, although not statistically significant. Also when analysed on a continuous scale hsCRP levels were associated with (cardiovascular) mortality in patients with the CCR5 ins/ins genotype and not in patients carrying a deletion allele. These results suggest that CCR5 deficiency (implicated by one or two copies of a non-functional CCR5 gene)

attenuates the adverse effects of a persistent inflammatory state that may lead to mortality in ESRD patients. Considering the importance of the inflammatory state for prognosis and the current developments in pharmacotherapy this finding may have considerable potential clinical implications.

Blocking CCR5 has been proposed as a novel therapeutic approach for cardiovascular conditions by interfering with systemic inflammation. This concept is supported by an animal study by Veillard et al. in which treatment of hypercholesterolemic mice with the CCR5 antagonist Met-RANTES reduced progression of atherosclerosis.[30] Moreover, Schober et al. demonstrated that treatment of apoE deficient mice with Met-RANTES reduced neo-intimal plaque area and macrophage infiltration.[31] Finally, treatment with TAK-799, a CCR5 chemokine receptor antagonist, reduced lesion development in a collar-induced carotid artery atherosclerosis model.[32]

To our knowledge, this is the first study investigating the interaction between CCR5 genotype, elevated (hs)CRP levels and (cardiovascular) mortality in dialysis patients. In line with our data, studies in other populations generally have shown an association of CCR5Δ32 with a favourable cardiovascular outcome, albeit not invariably so.[33] In males the presence of CCR5Δ32 is associated with reduced incidence of myocardial infarction at a younger age.[19] Another study suggested that the CCR5Δ32 genotype protected against coronary heart disease.[21] In the Nurses' Health study a possible association between the CCR5Δ32 polymorphism and a reduced incidence of early onset coronary heart disease was found.[20] Our data extend these findings in a population with a particularly high cardiovascular mortality, namely ESRD, showing that CCR5Δ32 attenuates the risk of mortality in patients with systemic inflammation as determined by a high hsCRP. Replication in an independent, somewhat different ESRD population supports the robustness of this finding. In patients without systemic inflammation the CCR5Δ32 had no effect on mortality risk, strongly suggesting that the CCR5Δ32, possibly by receptor deficiency, ameliorates the downstream effects of systemic inflammation.

Our data elicit the hypothesis that CCR5Δ32 modulates inflammation-driven atherosclerosis. Whereas our study does not allow any conclusion on the underlying mechanisms of the impact of CCR5Δ32 on inflammation driven mortality, several inferences can be made. Chemokines play an important role in the recruitment of inflammatory cells mediated through chemokine receptors. The presence of a dysfunctional CCR5 chemokine receptor can modulate inflammation. This was first described in HIV infected persons. Homozygous carriers of the CCR5Δ32 polymorphism were protected against HIV infection whereas heterozygotes showed a delayed progression to AIDS compared to non-carriers.[34-36] As inflammation is involved in atherogenesis,

it has been suggested that CCR5 ligands, CCR5 and genetic variation in CCR5 could play a role in the pathogenesis of vascular disease.[10-14, 30, 37-40] Thus, it can be hypothesized that CCR5 deficiency in carriers of the CCR5Δ32 polymorphism could be beneficial during an enhanced state of vascular inflammation. We used elevated hsCRP levels as an indicator of the state of (vascular) inflammation in our population, a phenotype that was shown to be a predictor of CVD and mortality in dialysis patients.[5, 6, 25] Little is known about the relationship between CCR5 or its ligands and CRP during ESRD or dialysis. However, in one study, it has been observed that acute transcription of anti-inflammatory cytokines following HD was significantly lower in patients with high serum CRP levels.[41] In contrast, transcription of pro-inflammatory cytokines (including IL-1β and TNF-α) was induced in equal concentrations, regardless of baseline CRP levels. IL-1β and TNF-α are known to induce expression of the CCR5 ligands CCL4 and CCL5 in renal disease.[42, 43] Thus, the diminished up regulation of anti-inflammatory cytokines, together with enhanced expression of cytokines that stimulate CCR5 mediated inflammation, could underlie the observed hsCRP-dependent increased mortality in dialysis patients homozygous for a functional CCR5 gene in our study.

A potential limitation of our study is that we were not able to study all included patients from the NECOSAD cohort, because data on hsCRP and/or CCR5 were not available in a subset of the patients. In this subset, mortality rate was slightly higher than in the patients available for analysis. In a single-cohort study, selection bias and population stratification cannot be excluded, despite the presence of Hardy-Weinberg equilibrium, and despite confirmation of results when only the Caucasian subjects were analysed. However, independent replication on a Swedish cohort, that was in Hardy Weinberg equilibrium as well, and in which data were available for all patients confirmed our results, hereby showing the robustness of our findings. As, moreover, the number of patients in the CCR5Δ32 groups was small, we did an analysis on the two cohorts combined, leading to the same results.

A single value of hsCRP was used in our analysis. It is possible that the hsCRP level was elevated because of acute infectious reasons and not due to a persistent inflammatory state. To exclude patients with an acute infection the analyses were redone by excluding patients with a CRP$\geq$50mg/l. This resulted in comparable HRs for all-cause and cardiovascular mortality. For non-cardiovascular mortality the HRs were comparable with those found for cardiovascular mortality, thereby underlining the importance of the CCR5 genotype in chronic, inflammation driven mortality.

We used the previously used hsCRP cut-off point of 10mg/l to divide patients in a group with or without a chronic inflammatory state. This cut-off point could potentially have influenced our results. However, analyzing the

interaction between CCR5 genotype and CRP level with the median hsCRP level as cut-off did not alter the results. Moreover, when analyzing hsCRP as a continuous variable, the hsCRP level seemed to influence mortality and cardiovascular mortality in the CCR5 ins/ins genotype group and not in the CCR5Δ32 genotype group.

Adjustments in genetic association studies could potentially introduce interference in the causal pathway and thereby bias through overadjustment.[44] For this reason, we reported unadjusted hazard ratios in the manuscript. However, as we studied CCR5 as effect modificator of the association between CRP levels and mortality, and CRP levels can be affected by confounding variables, like cardiovascular disease, diabetes and dialysis modality, we also reported adjusted hazard ratios in the manuscript.

Finally, we only studied a single polymorphism. The observed effect does not necessarily causally implicate this particular polymorphism, but could be due to another variant in linkage disequilibrium with the studied deletion. This variant does not necessarily have to be located within the CCR5 gene, since patterns of linkage disequilibrium do not follow the patterns of genes in the genome. This is a point that deserves further investigation. However, our efforts as reported in the present study were not toward in-depth characterization of the gene locus, but rather to investigate whether the impact of the polymorphism, reported in the literature to be associated with mortality, was modified by inflammatory status.

In conclusion, our results indicate that CCR5 genotype modifies the prognosis of mortality associated with inflammation in incident dialysis patients. Data from the literature suggest that this could be due to CCR5 deficiency (implicated by one or two copies of a non-functional CCR5 gene). This could lead to attenuation of the adverse effects of a persistent inflammatory state that is involved in increased all-cause and cardiovascular mortality in dialysis patients. Recently CCR5 blockade has become feasible in humans.[45] Our data suggest that is may be worthwhile to study whether pharmacological blockade of CCR5 could have therapeutic benefits in dialysis patients with persistent inflammation.

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complications in renal disease. GENECURE is hosted by the Renal Genome Network (ReGeNet) project (www.regenet.eu), a pan European network of clinicians and scientists from academia and industry seeking to generate and facilitate genetic and genomic studies to the clinical benefit of the renal patient.

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Chapter 4

TNF-α levels are not increased in inflamed patients carrying the CCR5 deletion 32

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Abstract

Recently we reported on a genetically predisposed protection from C-reactive protein (CRP) related mortality in dialysis patients carrying the functional CC-chemokine receptor 5 deletion 32 allele (CCR5Δ32) mutation. Since CCR5Δ32 is associated with a less pro-inflammatory immune response in mice, we hypothesized that the observed protection is (in part) due to a less pro-inflammatory cytokine profile.

We conducted a cross-sectional observational study including 263 incident dialysis patients aged 18-70 yrs, without clinical signs of infection and/or acute vasculitis. TNF-α, IL-6, IL-10 and hsCRP levels were determined and studied in relation to the CCR5 genotype.

We showed that in the presence of elevated hsCRP, IL-6 concentration was higher irrespective of the CCR5 genotype. However, in patients with the CCR5 deletion, TNF-α did not differ in the presence/absence of elevated hsCRP and was not correlated with hsCRP levels in carriers of the CCR5Δ32 polymorphism.

So, a possible underlying mechanism of the impact of CCR5Δ32 genotype on inflammation driven mortality in dialysis patients could be a reduced Th1 immune response as represented by decreased TNF-α levels.

Introduction

We recently reported on a genetically predisposed protection from C-reactive protein (CRP) related (cardiovascular) mortality in dialysis patients carrying the functional CC-chemokine receptor 5 deletion 32 allele (CCR5Δ32) mutation.[1] The CC-chemokine receptor 5 (CCR5) is expressed on monocytes and T-lymphocytes, especially Th1 cells.[2] A common genetic polymorphism consisting of a 32-basepair deletion in the open reading frame results in functional CCR5 deficiency by absence of CCR5 membrane expression. Heterozygous subjects express a lower amount of functional receptors compared to wild-type homozygotes.[3] Consistent with our findings, the CCR5Δ32 polymorphism has been associated with better (cardiovascular) outcome in different populations.[4-7] Whereas Th1 responses, as well as the cytokines TNF-α and INF-γ are pro-inflammatory, as opposed to Th2 responses and IL-10.[8, 9] IL-6 exhibits both pro- and anti-inflammatory effects.[10] Of the vast family of cytokines, TNF-α, IL-6 and IL-10 seem to play a major role in the development of Th1/Th2 imbalance, leading to increased (cardiovascular) complications and worse outcome in end stage renal disease (ESRD) patients.[11] In mice it has been shown that CCR5 deficiency is associated with reduced Th1 type responses, less TNF-α and INF-γ production and increased secretion of the anti-inflammatory cytokine IL-10.[12-15]

In this analysis, we hypothesize that the previous observed protection from CRP related (cardiovascular) mortality in CCR5Δ32 polymorphism carriers is (in part) due to a less pro-inflammatory immune response in the carriers of the deletion. We tested this in a cohort of incident dialysis patients in whom levels of CRP, TNF-α, IL-6 and IL-10 were assessed and related to their CCR5Δ32 genotype.

Patients and Methods

Study population

This analysis is framed within a prospective follow-up study in well-characterized chronic kidney disease (CKD) stage 5 patients sampled close to the start of renal replacement therapy from Karolinska University Hospital at Huddinge, Sweden, including patients of 18-70 years of age, without clinical signs of infection and/or acute vasculitis at time of admission. A detailed description was published previously.[16] This cohort was used as independent replication in our previous study about the modification of CCR5Δ32 on the association of CRP levels with mortality in ESRD patients. Diabetes and clinical history of cardiovascular disease (CVD) was recorded. Patients were divided in four groups according to their CCR5 genotype and

presence or absence of systemic inflammation defined by a high-sensitivity CRP (hsCRP) level >10 mg/l.[1]

Laboratory analyses

hsCRP, cholesterol, S-albumin and hemoglobin were analyzed using routine methods at the Karolinska University Hospital. Serum concentrations of TNF-α, IL-6 and IL-10 were quantified on the Immulite automatic analyzer (Diagnostic Products Corporation, Los Angeles, California, USA). Genotype analyses were performed by PCR amplification followed by size separation of the resulting fragments on a 3% agarose gel and visualization by ethidium bromide staining. The resulting fragments were 241 and 209 bp for the insertion and the deletion allele, respectively.

Statistical analyses

Hardy-Weinberg equilibrium was calculated using the gene-counting method. Differences between groups were tested with the chi-square test for dichotomous and categorical variables and one-way ANOVA for continuous variables. Spearman rank was used to test correlation. All statistical analyses were performed with SPSS statistical software (version 16.0; SPSS, Chicago, IL).

Results

Of 302 incident dialysis patients genotyped for CCR5, hsCRP level TNF-α, IL-6 and IL-10 levels were available in 263. No major differences were observed between the 263 included patients and those 39 with missing cytokine values. The CCR5Δ32 genotype was distributed as follows: ins/ins 218 (82.9%), ins/Δ32 41 (15.6%) and Δ32 /Δ32 4 (1.5%) and did not deviate significantly (p=0.21) from Hardy-Weinberg equilibrium. Baseline characteristics of CCR5Δ32 carriers and non-carriers are given in the Table 1. There were no differences in baseline characteristics between the two groups.

No significant differences in TNF-α, IL-6 and IL-10 levels were observed between the genotype groups. However, when evaluating these cytokines stratified by the CCR5 genotype and hsCRP level, significant differences were observed for TNF-α and IL-6 but not for IL-10 levels (Table 2). In the presence of elevated hsCRP, IL-6 concentration was higher irrespective of the CCR5 genotype ($p<0.01$). However, in patients with the CCR5 deletion, TNF-α did not differ in the presence/absence of elevated hsCRP (p=0.81). To further investigate this finding univariate correlations between TNF-α, IL-6 and hsCRP levels were studied (Table 3). As expected, whereas IL-6 was strongly associated with hsCRP (Rho=0.62) the correlation between hsCRP

and TNF-α was modest (Rho=0.26). When patients were stratified by the CCR5 genotype, no association between hsCRP and TNF-α was observed among patients carrying the CCR5Δ32 polymorphism indicating that TNF-α levels are not elevated in patients carrying the deletion despite a higher state of inflammation.

Table 1: Baseline characteristics according to CCR5 genotype. Data are presented as number (percentage), median (25, 75 percentile), mean (SD). GFR: glomerular filtration rate. No significant differences were observed between the groups.

	Non-del genotype N=218	**Del genotype** N=45
Gender: male	129 (59.2)	32 (71.1)
Age (year)	55 (44-63)	54 (46-65)
GFR (ml/min)	6.5 (2.2)	6.6 (2.8)
Cardiovascular disease (%)	72 (33.0)	12 (26.7)
Diabetes mellitus (%)	69 (31.7)	12 (26.7)
Cholesterol (mmol/l)	5.3 (1.5)	5.3 (1.3)
Hemoglobin (g/l)	104 (14)	105 (16)
Albumin (g/l)	32.9 (6.1)	33.6 (6.3)
hsCRP (mg/l)	4.6 (2.1-14.0)	6.7 (2.2-18.0)
TNF-α (pg/ml)	10.3 (8.0-13.5)	9.2 (7.8-11.4)
IL-6 (pg/ml)	6.4 (3.5-10.9)	6.5 (3.7-9.1)
IL-10 (pg/ml)	0.9 (0.9-1.6)	0.9 (0.9-1.6)

Table 2: Levels (median, 25 and 75 percentile) of TNF-α, IL-6 and IL-10 according to CCR5-hsCRP stratification. * Significantly (P<0.01) different from the matching uninflamed group.

	CCR5 ins/ins & hsCRP≤10 mg/L n=147	CCR5 del/ & hsCRP≤10 mg/L n=26	CCR5 ins/ins & hsCRP>10 mg/L n=71	CCR5 del/ & hsCRP>10 mg/L n=19
TNF-α (pg/ml)	9.6 (7.5-12.6)	8.7 (7.5-11.9)	12.2 (9.1-15.2)*	10.4 (7.9-11.4)
IL-6 (pg/ml)	4.6 (2.9-7.3)	3.8 (2.9-5.1)	11.1 (7.1-16.3)*	9.1 (8.1-15.6)*
IL-10 (pg/ml)	0.9 (0.9-1.4)	0.9 (0.9-1.2)	1.4 (0.9-2.9)	1.0 (0.9-2.6)

Discussion

This prospective study in incident dialysis patients suggest that our previous finding of an associating between mortality and elevated serum hsCRP concentrations is modulated by the CCR5Δ32 polymorphism could be mediated via less TNF-α elevation in inflamed patients carrying the CCR5Δ32 polymorphism. Whereas IL-6 was elevated in inflamed patients irrespective of their genotype IL-10 levels did not differ between the groups.

TNF-α is the classical pro-inflammatory cytokine with a pivotal role in the regulation of other inflammatory mediators. Elevated serum levels of TNF-α are associated with increased mortality and atherosclerosis in dialysis patients.[11, 17-19] The current observation that the association between an inflammatory trigger, as indicated by elevated hsCRP, and elevation of TNF-α is modulated by CCR5Δ32 is in accordance with animal reports where CCR5 deficiency was associated with reduced Th1 type responses, less TNF-α and INF-γ production; opposite to the animal data in this study IL-10 levels were not increased in human carriers of the CCR5Δ32 genotype.[12-15]

Acknowledged limitations in this study are the relatively small sample size and the existence of some data exclusions due to missing cytokine measurements. Besides this only patients from the validation cohort are incorporated in this study due to absence of cytokine levels in the other cohort used in our previous study.[1]

In conclusion, our results suggest that one underlying mechanism of the impact of CCR5Δ32 genotype on inflammation driven mortality in ESRD patients might be a reduced Th1 immune response trough less TNF-α production. This hypothesis needs to be tested in larger patient groups.

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Chapter 5

CCR5Δ32 genotype leads to a Th2 type directed immune response in ESRD patients

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Abstract

Background

In patients with end stage renal disease (ESRD) we observed protection from inflammation-associated mortality in CCR5Δ32 carriers, leading to CCR5 deficiency, suggesting impact of CCR5Δ32 on inflammatory processes. Animal studies have shown that CCR5 deficiency is associated with a more pronounced Th2 type immune response, suggesting that in human CCR5Δ32 carriers the immune response may be more Th2 type directed. So, in the present study we determined the Th1-Th2 type directed immune response in ESRD patients carrying and not carrying the CCR5Δ32 genetic variant after stimulation.

Methodology/Principal Findings

We tested this hypothesis by determining the levels of IFN-γ and IL-4 and the distribution of Th1, Th2 and Th17 directed circulating CD4+ and CD8+ T cells and regulatory T cells (Tregs) after stimulation in ESRD patients with (n=10) and without (n=9) the CCR5Δ32 genotype. The extracellular levels of IFN-γ and IL-4 did not differ between CCR5Δ32 carriers and non carriers. However, based on their intracellular cytokine profile the percentages IL-4 secreting CD4+ and CD8+ T cells carrying the CCR5Δ32 genotype were significantly increased (p=0.02, respectively p=0.02) compared to non carriers, indicating a more Th2 type directed response. Based on their intracellular cytokine profile the percentages IFN-γ and IL-17 secreting T cells did not differ between carriers and non-carriers nor did the percentage Tregs, indicating that the Th1, Th17 and T regulatory response was not affected by the CCR5Δ32 genotype.

Conclusions/Significance

This first, functional human study shows a more pronounced Th2 type immune response in CCR5Δ32 carriers compared to non carriers. These differences may be involved in the previously observed protection from inflammation-associated mortality in ESRD patients carrying CCR5Δ32.

Introduction

Genetic variability in the chemokine cascades could potentially influence disease outcomes by modifying inflammatory processes. CC-chemokine receptor 5 (CCR5) is one of the chemokine receptors. It is expressed on T cells and monocytes and it is important for recruitment.[1,2] Several polymorphisms have been described for CCR5. The CCR5Δ32 genetic variant is located on the chromosome 3p21 and consists of a 32-basepair deletion in the open reading frame. It effectively results in functional CCR5 deficiency by absence of CCR5 membrane expression.[3] We observed protection from inflammation-associated mortality in carriers of the deletion 32 allele in end stage renal disease (ESRD), suggesting impact of CCR5Δ32 on the inflammatory process of atherosclerosis.[4] Also in other human populations, characterized by high cardiovascular risk, the presence of the CCR5Δ32 genotype has been associated with better outcome.[5-8]

In chronic inflammatory processes like atherosclerosis T cells play an important role. Both CD4+ T cells and to a lesser extent CD8+ T cells are present in atherosclerotic lesions.[9-12] CD4+ T helper cells can differentiate into three effector lineages based on their cytokine expression: IFN-γ/TNF-α producers (Th1), IL-4 producers (Th2) and IL-17 producers (Th17).[12,13] In addition, a small fraction of CD4+ T cells can develop into cells with a regulatory function (Tregs) that are defined by their co-expression of high levels of surface CD25 and intracellular transcription factor forkhead box P3 (FoxP3). These Tregs have the remarkable ability to suppress the proliferation and effector function of other T cells.[9,12] As with CD4+ T cells, CD8+ T cells can differentiate to T cytotoxic (Tc)1 or Tc2 cell subsets, secreting predominantly Th1 or Th2 cytokines respectively.[13]

Atherosclerotic inflammation is regarded as a (partly) Th1 driven condition.[12] The CCR5 receptor is highly expressed T-lymphocytes, on both CD4+ T and CD8+ T cells.[2,11] In atherosclerotic mice CCR5 deficiency is associated with a more pronounced Th2 type immune response and less TNF-α and IFN-γ production hereby counteracting the Th1 directed Th1/Th2 disequilibrium of atherosclerotic inflammation.[14-17]

These data fuel the hypothesis that the immune response in carriers of the CCR5Δ32 genotype is more Th2 type directed. Such differences in response might play a role in the protection against inflammation-associated mortality in ESRD in carriers of the CCR5Δ32 genotype. To test this hypothesis we

studied the cell mediated immune responses in peripheral mononuclear cells (PBMCs) in ESRD patients. We first determined the extracellular levels of IFN-γ and IL-4 after stimulation of PBMCs, and second the distribution of Th1, Th2 and Th17 directed circulating CD4+ and CD8+ T cells, based on their intracellular cytokine profile after stimulation, and the percentage of Tregs in ESRD patients with and without the CCR5Δ32 genotype.

Methods

Objectives

The objective of the present study was to determine possible differences in cell mediated immune response between ESRD carriers and non carriers of the CCR5Δ32 genotype. To test this hypothesis we first determined the IFN-γ and IL-4 levels after stimulation of peripheral mononuclear cells (PBMCs) and secondly the distribution of Type-1, Type-2 and Type-17 directed circulating CD4+ and CD8+ T cells, based on their intracellular cytokine profile, as well as the frequency of FoxP3+ regulatory T cells.

Participants

Biosamples and data from twenty patients with ESRD were included in this study. These patients were part of an ESRD cohort from a single kidney transplant centre in the Netherlands (University Medical Center Groningen), in whom data and biosamples were collected prior to kidney transplantation. As part of a larger genotyping project, all patients were genotyped as described below. For the current project we randomly selected five homo- and five heterozygous carriers from the cohort. Ten wild type patients were matched with carriers according to time of inclusion, hereby creating similar preservation conditions.

Genotyping

The genotypes were determined with a PCR-based allelic discrimination assay using primers (Life Technologies) and allele-specific probes (PE Biosystems) as described previously.[18] Patients were grouped by CCR5 genotype, namely those homozygous for the major allele (non-carriers) and those with 1 or 2 deletion alleles (carriers). Patients with one or two deletion

alleles were grouped together, as it has been demonstrated that presence of one deletion allele is sufficient to compromise CCR5 function.[3]

Sample preparation and thawing

Heparinized venous blood was obtained from ESRD-patients who gave their informed consent. PBMCs were separated by conventional Ficoll gradient and frozen in 10% DMSO in FCS and stored in liquid nitrogen. PBMCs were thawed and washed twice with RPMI 1640 media (Cambrex Bio Science, Verviers, Belgium), supplemented with 10% heat inactivated fetal calf serum and 50 µg/mL gentamycin (Gibco, Scotland, UK).

Determination of extracellular cytokine by ELISA

Thawed PBMCs were cultured in a 5mL polypropylene tubes (BD Biosciences) at 2,5 $x10^6$ cells/mL per tube, and stimulated with 40 n*M* phorbol myristate acetate (PMA; Sigma-Aldrich, Steinheim, Germany) and 2 n*M* calcium ionophore (Sigma-Aldrich). Culture supernatants were collected over a period of 24 hours to determine the extracellular levels of IFN-γ, and IL-4 cytokines.

Cytokine levels of IFN-γ and IL-4 were measured by commercial sandwich enzyme linked immunoassay (ELISA) kits (Pelikine Compact, Sanquin, Amsterdam, The Netherlands), according to the instructions of the manufacturer.

Determination of intracellular cytokine by flow cytometry

The following conjugated antibodies were used in flow cytometry: allophycocyanin (APC)–Cy7–conjugated anti-CD69, peridin-chlorophyll protein (PerCP)–conjugated anti-CD8, phycoerythrin (PE)– Cy7–conjugated anti–IL-4, and Alexa Fluor 700–conjugated anti-IFN-γ (all from Becton & Dickinson, Amsterdam, The Netherlands). Alexa Fluor 488–conjugated anti–IL-17, Alexa Fluor 647-conjugated anti-TNF-α, and eFluor605™–conjugated anti-CD3 were obtained from eBioscience (San Diego, CA). To determine the frequency of T cell subsets by measuring intracellular cytokine production, cells were stimulated for 4 hours with 40 n*M* PMA and 2 n*M* calcium ionophore. Brefeldin A (10µg/mL) was added to inhibit cytokine release. After stimulation, cells were washed in wash buffer (phosphate buffered saline, 5% fetal bovine serum, 0.1% sodium azide; Merck,

Darmstadt, Germany) and stained with eFluor 605-conjugated anti-CD3, PerCP–conjugated anti-CD8 and APC-Cy7–conjugated anti-CD69 for 15 minutes at room temperature. Cells were fixed with 100 µl Reagent A (Caltag, An Der Grab, Austria) for 15 minutes. After washing, the pellet was resuspended in 100 µl permeabilization Reagent B (Caltag) and labeled with PE-Cy7–conjugated anti–IL-4, Alexa Fluor 700–conjugated anti-IFN-γ, Alexa Fluor 647-conjugated anti-TNF-α, Alexa Fluor 488–conjugated anti–IL-17, and APC-Cy7–conjugated anti-CD69 for 30 minutes in the dark. After staining, the cells were washed and immediately analyzed on a FACS-LSRII flow cytometer (Becton Dickinson). Seven-color flow cytometric acquisition was performed using FACSDiva software (Becton Dickinson). For all flow cytometry analyses, data were collected for 2 x 10^5 cells and plotted using the Win-List software package (Verity Software House, Topsham, ME). Because stimulation reduces surface expression of CD4 on T cells, CD4 T cells were identified indirectly by gating on CD3+ and CD8− lymphocytes, whereas CD8+ T cells were identified by directly gating on CD3+ and CD8+ lymphocytes. Subsets of activated CD4+ and CD8+ T cells in response to mitogenic stimulation were evaluated by double expression of activation marker CD69 and intracellular cytokine production of IFN-γ (for type-1) or IL-17 (for type-17) or IL-4 (for type-2). The unstimulated samples were used as a guide for setting the linear gates to delineate positive and negative populations.

Determination of regulatory T cell frequencies

PBMCs were washed with cold PBS (pH 7.2) and incubated with appropriate concentrations of PerCP-conjugated anti-CD4, FITC-conjugated anti-CD3 and PE-conjugated anti-CD25 (all purchased from BD) for 30 min at 4 °C in the dark. Cells were then washed with cold PBS, followed by fixation and permeabilization in Fix/Perm buffer (FoxP3 staining kit, eBioscience, Uithoorn, The Netherlands) for 45 min at 4 °C. Subsequently, cells were washed with cold permeabilization buffer (FoxP3 staining kit, eBioscience, Uithoorn, The Netherlands). To block nonspecific binding, normal rat serum was added for 10 min, followed by the addition of APC-conjugated rat anti-human FoxP3 (eBioscience, Uithoorn, The Netherlands). After incubation for 30 min at 4 °C, the cell suspension was washed twice with cold permeabilization buffer, and immediately analyzed on FACS-Calibur (BD). Data were collected for 2 x 10^5 cells and plotted using the Win-List software package (Verity Software House, Topsham, ME). Positively and negatively

stained populations were calculated by quadrant dot-plot analysis, determined by the isotype matched control antibodies of irrelevant specificity (obtained from BD and eBioscience).

Ethics

All patients gave written informed consent and the local medical ethics committee from the University Medical Center Groningen (METc UMC Groningen), the Netherlands, gave their approval.

Statistical methods

Patients were grouped by CCR5 genotype, namely those homozygous for the major allele (non-carriers) and those with 1 or 2 deletion alleles (carriers). The latter were grouped together, as it has been demonstrated that presence of one deletion allele is sufficient to compromise CCR5 function.[3] Data are presented as the median. The nonparametric Mann-Whitney U test was used to compare data from patients with and without the CCR5Δ32 genotype. Differences were considered statistically significant at 2-sided *p* values less than 0.05.

Table 1: Baseline characteristics

	CCR5 wild type (n= 9)	**CCR5 deletion 32** (n=10)
Gender; male	4 (44.4)	7 (70.0)
Age (year)	51 (14)	51 (13)
Primary kidney disease		
Renal vascular disease and hypertension	3	3
Diabetes mellitus	1	0
Cystic kidney disease	1	3
Pyelonephritis	1	1
Primary oxalosis	1	0
Unknown	2	3
Dialysis duration (days)	1570 (880)	1005 (734)
Hemodialysis	5 (55.6)	4 (44.4)
Body Mass Index	26 (4.94)	25 (3.21)
Systolic bloodpressure (mmHg)	147 (19)	138 (10)
Diastolic bloodpressure (mmHg)	86 (8)	86 (9)

Data are presented as number (percentage), mean (SD)

Results

Patients

From the 20 patients who were included in this study 1 stimulation test failed due to the fact that the T cells could not be stimulated. Further statistical analyses were performed on the remaining 19 patients. Baseline characteristics are shown in Table 1. There were no statistically significant differences in baseline characteristics between the 2 genotype groups.

Extracellular cytokines of type-1 and type-2 T cells

To assess the functional capacity of the responding PBMCs the total amount of IFN-γ and IL-4 after stimulation was determined. As shown in Figure 1, the levels of extracellular IFN-γ and IL-4 were not statistically significant different between carriers and non carriers of the CCR5Δ32 genotype.

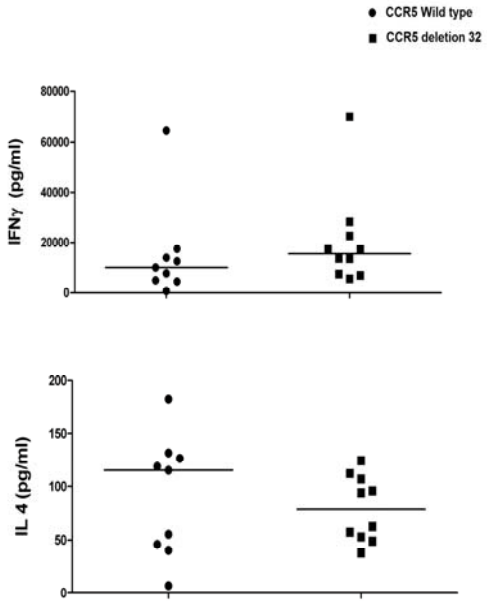

Figure 1: IFN-γ and IL-4 ELISA per 2,5 x 10^6 PBMCs in carriers and non carriers of the CCR5Δ32 genotype. Levels of IFN-γ and IL-4 after stimulation from carriers (n=10) and non carriers (n=9) are shown. Horizontal lines represent the medians.

Intracellular cytokines of type-1, type-2, and type-17 T cells

To elucidate the functional phenotype of the CD4+ and CD8+ T cells responding to stimulation, activated T cells were gated and evaluated for expression of the activation marker CD69 versus intracellular expression of the cytokines IFN-γ, IL-17 and IL-4. The results are shown in Figure 2 and 3. The percentages IL-4 secreting CD4+ and CD8+ T cells from patients with the CCR5Δ32 genotype was significantly (p= 0.02, respectively p= 0.02) increased compared to patients not carrying the CCR5Δ32 genotype, indicating a more Th2 type directed response. The percentages IFN-γ secreting CD4+ and CD8+ T cells did not significantly differ between carriers and non carriers of CCR5Δ32, meaning the Th1 and Th17 response did not differ between these 2 groups. Comparing the CCR5Δ32 homozygous and heterozygous CD4+ and CD8+ T cells showed no significant differences.

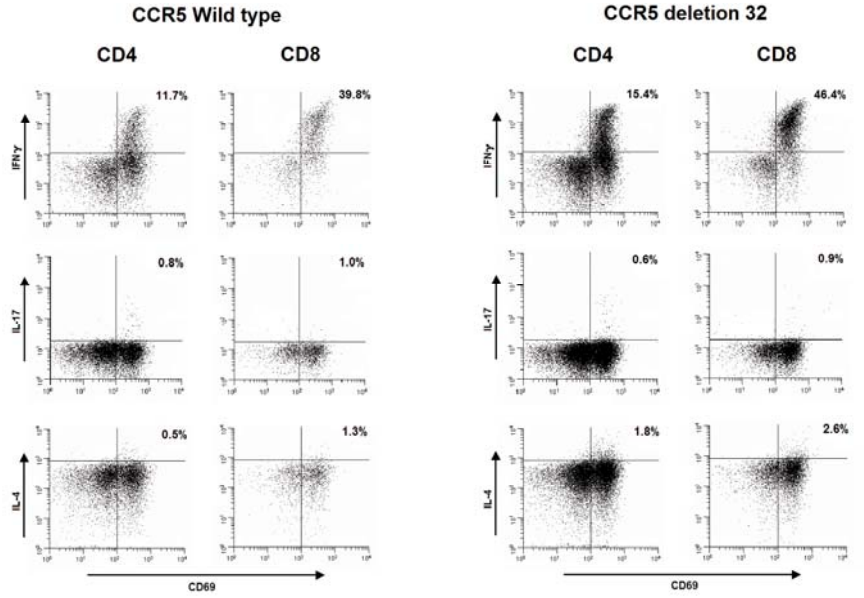

Figure 2: Flow cytometric characterization of CD4 and CD8 T cell subsets from CCR5 wild type (left panel) and CCR5 deletion 32 (right panel). PBMCs were stimulated *in vitro* with PMA and Ca-ionophore for 4 hours in the presence of BFA. The CD4 and CD8 T cell subsets were then assessed for the expression of activation marker CD69 versus intracellular cytokine (IFN-γ, IL-17, and IL-4). The percentage in the upper right corner of each plot represents the net percentage of positive cells.

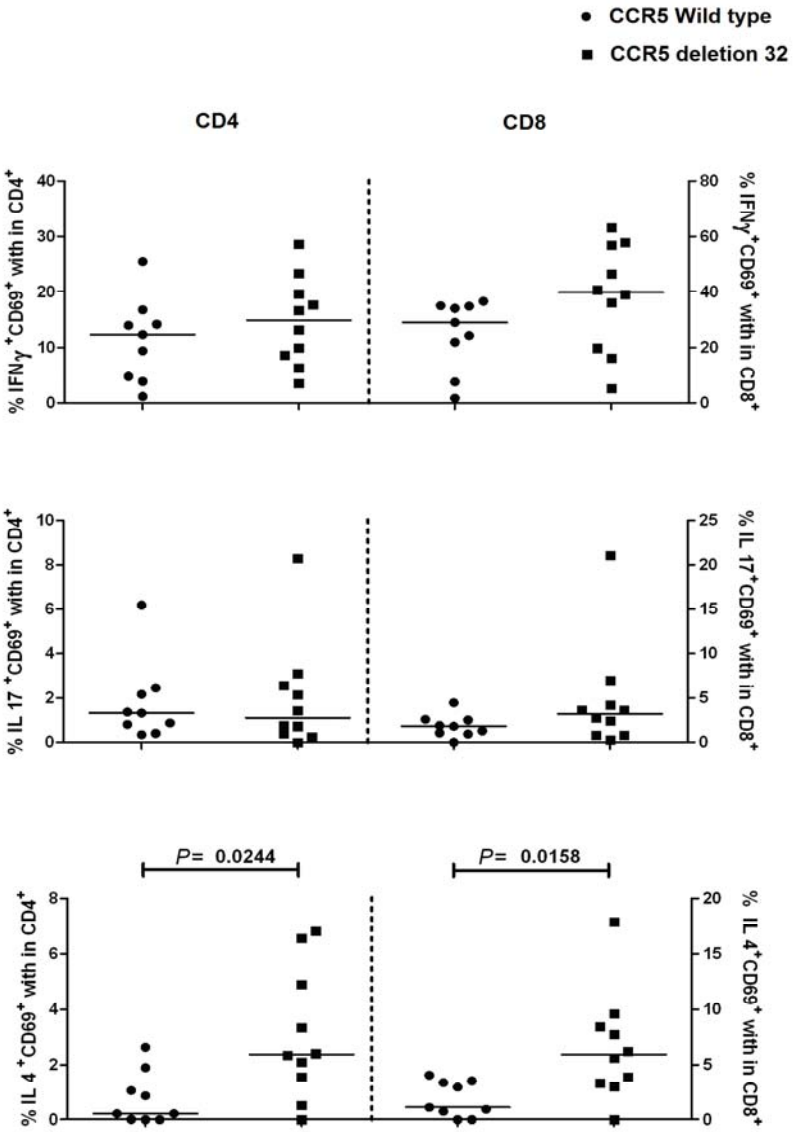

Figure 3: Percentages of IL-4, IL-17 and IFN-γ secreting CD4 and CD8+ T cells in carriers and non carriers of the CCR5Δ32 polymorphism. In the left panel the frequencies of IL-4, IL-17 and IFN-γ secreting cells among CD69+, CD4+ T cells from non carriers (n=9) and carriers (n=10) of the CCR5Δ32 genotype are shown. In the right panel the frequencies of IL-4, IL-17 and IFN-γ secreting cells among CD69+, CD8+ T cells from non carriers (n=9) and carriers (n=10) of the CCR5Δ32 polymorphism are shown. Horizontal lines represent the median percentage.

Frequencies of regulatory T cells

To address the question whether differences in Tregs frequencies influence the distribution of T cell subsets between CCR5Δ32 carriers and non carriers, FoxP3+CD25^{High}CD4^{+} T cells were analyzed in both groups. No significant differences were found between the 2 genotype groups (Figure 4). It seems, therefore, that Tregs are not responsible for the differences in Th2 response between CCR5Δ32 carriers and non carriers.

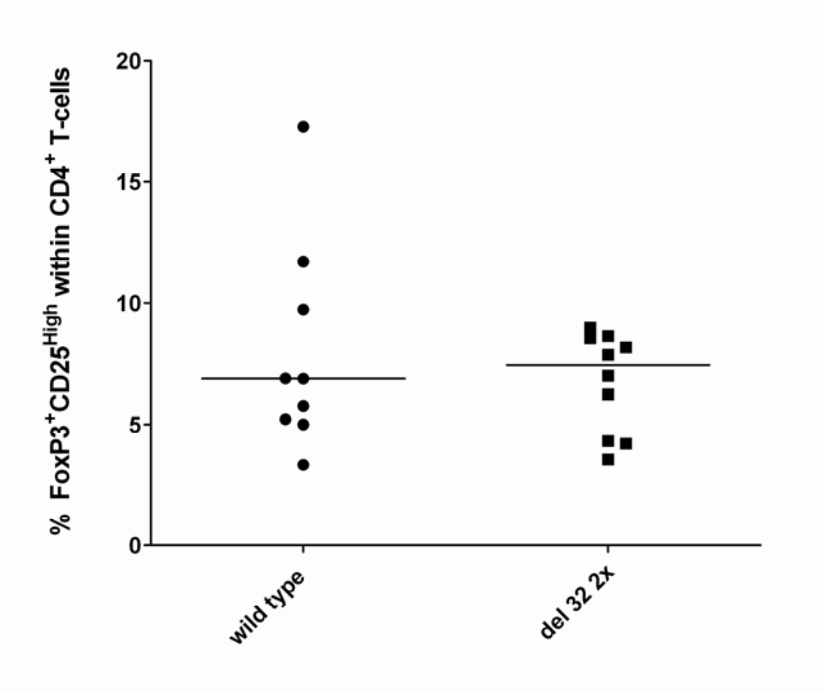

Figure 4: Percentages of Tregs (FoxP3^{+}CD25^{High} CD4^{+} T cells) in carriers and non carriers of the CCR5Δ32 polymorphism. Horizontal lines represent the median percentage.

Discussion

In the present study we demonstrate a skewing of circulating CD4+ and CD8+ T cells towards the Th2 phenotype based on their intracellular cytokine profile after stimulation in ESRD patients carrying the CCR5Δ32 genotype. These data are in line with animal data showing that genetic deficiency of CCR5 results in a shift in immune response towards a Th2 type response, and support the assumption that genetic differences in immune response are involved in the protection against inflammation-associated mortality in ESRD patients reported previously.[4]

In ESRD patients cardiovascular disease is a main cause of premature deaths.[19] Chronic inflammation is a major contributing factor.[20,21] The inflammatory nature of the process of atherosclerosis is nowadays well recognized.[22] In this process T cells play an important role.[9-12] Th1 cells, which produce IFN-γ as the principal cytokine, are thought to be pro-inflammatory and pro-atherogenic and are the most prevalent subtype in atherosclerotic lesions; Th2 cells, with IL-4 as the major cytokine, have the ability to inhibit Th1 differentiation and could therefore be anti-atherogenic. The role of Th17 cells, producing IL-17, in atherosclerosis is not yet clear.[23] So, until now atherosclerotic inflammation is regarded as a Th1 directed Th1/Th2 disequilibrium.[12]

To our knowledge, this is the first functional study investigating the Th1/Th2 directed immune response in relation to the CCR5Δ32 genetic variant in human. Animal studies consistently show a more pronounced Th2 immune response during genetic deficiency of CCR5 or pharmacological CCR5 blockade in atherosclerotic and other inflammatory conditions. In diet induced atherosclerotic inflammation in mice, genetic deletion of CCR5 was associated with a more stable plaque phenotype and reduced Th1 type immune response of stimulated splenocytes and an increased Th2 type response in splenocytes and lymph node cells.[14] After wire injury in mice with CCR5 deficiency a more atheroprotective immune response was seen, i.e. low IFN-γ and elevated IL-10 in CD4+ splenocytes compaired to CCR5 wild type mice.[15] Also in genetically CCR5 deficient mice with diet induced atherosclerosis, reduced lesion size, increased IL-10 and decreased TNF-α production by CD4+ and CD8+ T cells,[16] and reduced macrophage accumulation in plaques and lowered circulating IL-6 levels was seen.[17] In CCR5 deficient mice a more CD4+ Th2 cell activation pattern was seen in colitis in contrast to CCR5 wild type mice.[24] Interestingly, in CCR5

genetically deficient mice who received a renal allograft less Th1 associated markers and increased Th2 associated markers were found during chronic intragraft immune response.[25] In an islet transplantation model it was shown that in genetically CCR5 deficient mice not only in the intragraft immune response but also in the periphery a Th2 shift occurred.[26] In mice with diet-induced atherosclerosis treatment with a RANTES chemokine antagonist, hereby blocking CCR5, reduced atherosclerotic plaque formation, associated with reduced proliferation and secretion of Th1 cytokines IFN-γ and TNF-α, without difference in Th2 cytokine profile.[27] In rats pharmacological CCR5 blockade in stimulated endothelial cells inhibited selective transmigration of CD4+ Th1 cells.[28]

Our results extend these animal data on functional differences in immune response for the first time to a human setting and support our previous human cross-sectional association study, showing absence of association between serum CRP and TNF-α levels in ESRD patients carrying the CCR5Δ32 genotype, in contrast to patients without this genetic variant, supporting a reduced Th1 immune response in CCR5Δ32.[29] It should be emphasized that ELISA and flowcytometry methods give different types of results and that measuring the intracellular cytokine production by FACS is more accurate than ELISA. The measured cytokines by ELISA can be released from several cells, whereas FACS-method identifies the intracellular cytokines produced on a single-cell level. In addition, difference in T cell numbers between the study samples may influence the results obtained from ELISA but not from FACS method hereby probably explaining why we did not find a difference in extracellular cytokine levels between CCR5Δ32 carriers and non carriers. Since Tregs are responsible for regulation and suppression of T cell responses,[9,12] one may argue that differences in Tregs could underlie the increase in IL-4 expression in CCR5Δ32 carriers. However, no significant differences were observed in the percentages of Tregs between CCR5Δ32 carriers and non carriers. Thus, the increased Type 2 response in CCR5Δ32 carriers cannot be related to different frequencies of Tregs.

Together, these findings provide an explanation for the previously observed protection from inflammation-related mortality in ESRD in CCR5Δ32 carriers, as they support a less pro-inflammatory, pro-atherogenic immune response in carriers of the deletion.[4] Our results could also provide a mechanism underlying the protection against atherosclerosis by pharmacological

blockade of the CCR5 pathway in animal studies.[30-32] Of note, CCR5 blockade has become feasible in humans, and is currently used for treatment of HIV-infection.[33] It has been proposed that CCR5 blockade may be a strategy for protection against inflammation driven cardiovascular disease in ESRD and/or transplantation.[34,35] Our current results contribute to understanding of mechanisms that could be affected by CCR5 blocking agents in ESRD.

Limitations

Acknowledged limitation in this study is the relatively small sample size. Besides this one blood sample failed to be stimulated. However, as mentioned above, the results are in accordance with animal data and extend the findings of a correlation study in ESRD patients, supporting the robustness of our findings. Another limitation is that we studied ESRD patients only. ESRD as such affects T-cell properties.[36] Whereas the effects of CCR5 deficiency appear to be remarkably consistent across different species and inflammatory conditions, nevertheless generalization of our results to other populations would require a separate study.

In conclusion, we present the first human data on a difference in Th1/Th2 balance dependent on the CCR5Δ32 genotype in ESRD patients. Stimulated CD4+ and CD8+ T cells of patients with one or two CCR5Δ32 alleles show an increased Th2 type phenotype base on their intracellular cytokine profile. Differences in immune response may be involved in the impact of CCR5Δ32 on outcome in ESRD, and possible other inflammatory conditions.

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Chapter 6

Using a genetic, observational study as a strategy to estimate the potential cost-effectiveness of pharmacological CCR5 blockade in dialysis patients

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Abstract

Randomized clinical trials are expensive and time-consuming. Therefore, strategies are needed to prioritize tracks for drug development. Genetic association studies may provide such a strategy by considering the differences between genotypes as a proxy for a natural, lifelong, randomized at conception, clinical trial. Previously an association with better survival was found in dialysis patients with systemic inflammation carrying a deletion variant of the CC-chemokine receptor 5 (CCR5Δ32). We hypothesized that in an analogous manner, pharmacological CCR5 blockade could protect against inflammation driven mortality and estimated if such a treatment would be cost-effective.

A genetic screen-and-treat strategy was modelled using a decision-analytic Markov model, in which patients were screened for the CCR5Δ32 polymorphism and those with the wild type and systemic inflammation were treated with pharmacological CCR5 blockers. Kidney transplantation and mortality rates were calculated using patient level data. Extensive sensitivity analyses were performed.

The cost-effectiveness of the genetic screen-and-treat strategy was €18,557 per life-year gained and €21,896 per QALY gained. Concordance between the genetic association and pharmacological effectiveness was a main driver of cost-effectiveness. Sensitivity analyses showed that even a modest effectiveness of pharmacological CCR5 blockade would result in a treatment strategy that is good value for money.

Pharmacological blockade of the CCR5 receptor in inflamed dialysis patients can be incorporated in a potentially cost-effective screen-and-treat program. These findings provide formal rationale for clinical studies. This study illustrates the potential of genetic association studies for drug development, as a source of Mendelian randomized evidence from an observational setting.

Introduction

Pharmacological interventions that are of benefit in non-dialysis populations have thus far been disappointing in dialysis patients, underscoring the need for novel intervention strategies, specifically targeted at the dialysis population.[1, 2] However, development of novel pharmacological approaches followed by randomized clinical trials is expensive and time consuming, providing an immense obstacle to the development and introduction of innovative approaches in patient care. Research and development costs for a single approved cardiovascular drugs can reach hundreds of millions of dollars, with most costs accrued in phase II and III trials.[3] Therefore, alternative strategies are urgently needed to facilitate the multi-faceted process from drug development to introduction in clinical practice. Observational studies using genetic variants might provide such a strategy.[4] Given the random assignment of alleles in gamete formation, genetic variants can be considered to mimic the randomization process of randomized clinical trials. Data obtained through genetic association studies could therefore be considered a type of natural, lifelong, clinical trial, with genetically different groups being randomized at conception, hereby limiting confounding. This approach is known as Mendelian randomization.[5, 6]

One of the main driving forces in the accelerated atherosclerosis in end stage renal disease (ESRD) patients is chronic inflammation.[7] This population might therefore benefit from alternative therapies directed against the chronic inflammatory response. In this inflammatory process chemokines and chemokine receptors play an important role.[8-10] One of the chemokine receptors involved is the CC-chemokine 5 receptor (CCR5). Animal data show that pharmacologic intervention in the CCR5 chemokine pathway reduces atherosclerosis.[11-13] The relevance of these findings for humans is supported by genetic association studies on the CCR5 deletion 32 (CCR5Δ32) polymorphism, leading to functional CCR5 deficiency.[14] These studies show that CCR5Δ32 is associated with better outcome in different populations.[15-18] Previously, we found that CCR5Δ32 was associated with protection against mortality in Dutch cohort of dialysis patients characterized by inflammation and replicated these findings a in Swedish cohort.[19] Taken together, these data suggest that intervention targeting inflammation, in particular targeting the CCR5, may have the potential to improve prognosis in ESRD.[20]

Interestingly, pharmacological blockade of CCR5 is feasible in human as it is applied in clinical practice for treatment of HIV infection, which increases the feasibility of development of CCR5 blockade as a treatment strategy for protection against inflammation-driven atherosclerosis in ESRD.[21]

In line with the above, genetic association data on long term outcome in patients with versus without CCR5Δ32 can be considered as a virtual long term randomized intervention study on pharmacological blockade of the CCR5 receptor providing a fast and cheap simulation set-up for a real-life clinical trial. Systematic reviews have shown that pharmacogenetic screen-and-treat programs show great potential for developing cost-effective treatment modalities.[22, 23] In the current analysis, we use these concepts to estimate the potential cost-effectiveness of CCR5Δ32 screening and pharmacological CCR5 blockade in dialysis patients, from the perspective of the Dutch health-care system.

Methods

Patients

For the present study we used data from our previously published study on the effect of the CCR5Δ32 polymorphism on inflammation associated mortality in dialysis patients. This study was part of the NEtherlands COoperative Study on the Adequacy of Dialysis (NECOSAD), a multicenter prospective follow-up study comprising incident (new and consecutive) ESRD patients from 38 Dutch dialysis centers included between July 1998 and December 2001. Detailed descriptions of the study design and results have been published previously.[19]

Eligibility criteria for inclusion in the NECOSAD cohort were 18 years or older and no previous renal replacement therapy. All patients gave informed consent and all local medical ethics committees gave their approval. Patients were evaluated at 3 and 6 months after start of dialysis and every 6 months thereafter until death or date of censoring. Censoring involved transfer to a non-participating dialysis center, withdrawal from the study or end of the follow-up period in June 2007. Patients receiving a kidney transplant were not censored; data on their survival were obtained from the Dutch renal registry (RENINE).

Data collection and clinical definitions

High sensitivity CRP (hsCRP) was measured by means of particle-enhanced immunonephelometry using a standard CardioPhase hsCRP for BNII (Dade Behring Holding GmbH, Liederbach, Germany; detection limit 0.1 mg/l, precision 0.1 mg/l).[24] Systemic inflammation was defined as hsCRP concentrations above 10 mg/l. This cut-off point has been used in ESRD patients and has been validated with regard to the prediction of survival of ESRD patients.[25] Also it was demonstrated that a single measurement of elevated CRP levels was associated with a similar predictive power on mortality as repeated CRP measurements.[26]

CCR5 genotypes were determined with a PCR-based allelic discrimination assay using primers (Life Technologies) and allele-specific probes (PE Biosystems) as described previously.[27]

Patients were divided in 4 groups based on their CCR5Δ32 genotype and hsCRP level: CCR5 ins/ins with low hsCRP (≤10 mg/l), CCR5 ins/ins with high hsCRP (>10 mg/l), CCR5Δ32 with low hsCRP (≤10 mg/l) and CCR5Δ32 with high hsCRP level (>10 mg/l). Patients homo- or heterozygous for the deletion-allele were clustered since the presence of one minor allele has been associated with reduced receptor function.[14] Causes of death were classified according to the codes of the European Renal Association – European Dialysis and Transplantation Association (ERA-EDTA).[28] The following codes were used to classify cardiovascular mortality: myocardial ischemia and infarction; cardiac failure, fluid overload and pulmonary oedema; cardiac arrest; cerebro-vascular accident; haemorrhage from ruptured vascular aneurysm; mesenteric infarction; hyperkalaemia; hypokalaemia; cause of death uncertain or unknown.

Analytical approach

We modelled the potential cost-effectiveness of CCR5Δ32 screening and pharmacological CCR5 blockade using a decision-analytic Markov model (Figure 1). Markovian modelling is a commonly used technique in decision analyses to handle the complexity of multiple interconnective possible consequences.[29] The health states in our Markov model were hemodialysis (HD), peritoneal dialysis (PD), renal transplantation (Tx) and death. Cohorts of 1000 patients entered the model in the HD or PD health-state and were followed for a time period of 10 years.

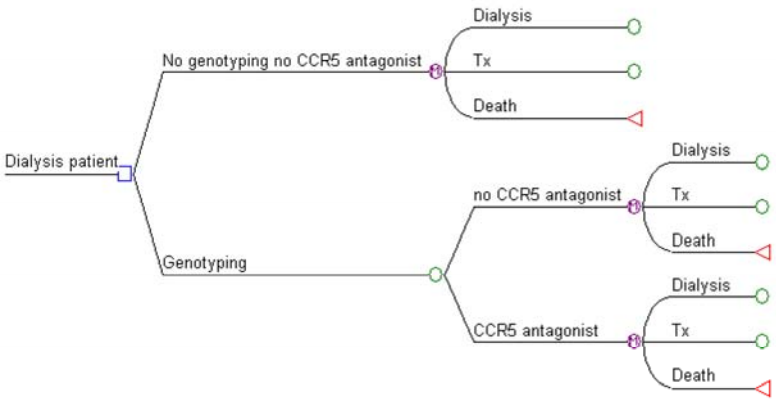

Figure 1: Decision tree and Markov model (M). Transition probabilities of the Markov model are shown in table 2.

Clinical data were used to model transition probabilities; patients could receive a kidney transplant, experience renal graft failure and return to dialysis or die. The number of patients in each health state was determined by monthly cycles throughout the entire follow up period.[30]

Effectiveness of pharmacological CCR5 blockade

Transition probabilities for kidney transplantation and mortality were calculated using the patient level NECOSAD data.[19] Kidney transplantation and mortality rates were calculated for the four patient groups. Because of small numbers the rate of renal transplant failure was calculated for all four groups combined. Pharmacological CCR5 blockade was assumed to mimic the effects of the Δ32 polymorphism in subjects with high inflammation status, thus improving patient survival in the patient group with the CCR5 ins/ins genotype and systemic inflammation up to the level of the patient group with the CCR5Δ32 polymorphism and systemic inflammation. In particular, the relative risk (RR) for pharmacological CCR5 blockade in the inflamed group was calculated using clinical data as 0.61 for all-cause mortality, 0.41 for cardiovascular mortality and 0.80 for non-cardiovascular mortality. While the main focus of the current analysis was on mortality, we also calculated, based on clinical data, that pharmacological CCR5 blockade improved the probability of renal transplantation (RR=2.41). To reflect our

main focus on mortality we performed a separate analysis without modelling an effect on the probability of renal transplantation.

Utilities

Health-related quality of life (QoL) of patients on haemodialysis (HD) and peritoneal dialysis (PD) were obtained by interviewing patients participating in the NECOSAD study, detailed inclusion criteria and methods are described elsewhere.[31] QoL of patients in the Dutch NECOSAD study were assessed with the EQ-5D instrument, which were applied to data from a UK population sample to obtain community based preference data.[32] No QoL-assessment of transplanted patients was performed in NECOSAD patients; these utilities were obtained from a Swedish study.[33] With QoL measurements, cost-effectiveness estimations can be made in terms of costs per Quality-adjusted Life-years (QALY) gained. A commonly cited implicit thresholds for treatments that are deemed good value for money is €50,000 per QALY in The Netherlands.[34]

Costs

A third-party health-care payer perspective was adopted for cost estimates. Health-care costs were classified into one of two categories: related costs and unrelated future costs.[35]

Related costs comprise costs directly related to the strategy under consideration. The cost of the genetic screening test for the CCR5Δ32 polymorphism was based on polymerase chain reaction and included staff costs.[36] The price of hsCRP screening was based on Dutch laboratory prices. Drug costs of pharmacological CCR5 blockade were based on Dutch prices of the CCR5 antagonist Maraviroc 300 mg (Celsentri) once daily,[37] including 6% value-added tax and a three-monthly pharmacists' prescription fee of €6,00. Costs of cardiovascular mortality were based on national Dutch life tables and health-care expenditures adjusted for comorbidities.[38] Costs of non-cardiovascular death and of transplantation graft failure were derived from a study with data from Dutch registries on renal diseases.[39]

Unrelated future costs comprised costs that are independent of current spending, apart from the effects of that spending on survival.[40, 41] In particular, as dialysis and renal transplantation care are not a direct consequence of CCR5 blockade but of the preexisting condition of end-

stage renal disease; these costs were consistently classified as unrelated future costs. The costs of dialysis and renal transplantation were based on data on volumes of recourse use, including consultations, hospitalisations and laboratory services and use of medication obtained from the NECOSAD study.[31]

In line with current pharmacoeconomic guidelines, unrelated future costs were not included.[35, 42] However, to determine the influence of unrelated future costs, these costs were included in a separate analysis. All costs were updated to 2009 values.

Discounting rates

Costs were discounted at 4% per annum and health effects at 1.5% per annum, following Dutch guidelines for pharmacoeconomic research.[43]

Sensitivity analyses

Univariate and probabilistic sensitivity analyses and a threshold analysis were performed. In the univariate sensitivity analysis, all model parameters were varied by 25% in order to determine the main cost and effect drivers in our model. Discount rates were varied to 0% and 3% per annum based on recommendations by Gold et al and Drummond et al.[44] The probabilistic sensitivity analysis was performed according to standard methods,[29] using 10,000 iterations and included all model parameters, except therapy costs and effectiveness of pharmacological CCR5 blockade which were explored in a threshold analysis. Gamma distributions were assumed for costs and beta distributions for utilities.[29] In the absence of data on standard deviations for costs, we assumed 25% of the mean. Uncertainty in mortality and transplantation rates was captured by nonparametric bootstrapping of the NECOSAD data, using 10,000 iterations.[45] As equivalence between genetic effects and associated pharmacologic effectiveness is not a given fact,[46] a threshold analysis was performed to determine the combined influence of drug effectiveness and treatment costs of pharmacological CCR5 blockade on the cost-effectiveness of the screen-and-treat strategy. The pharmacoeconomic model and sensitivity analyses were constructed using the statistical package R, version 2.5.1. A graph of the threshold analysis was constructed using Sigmaplot version 10.0.

Results

Study population

The study population used for modeling consisted of 413 patients. The CCR5 ins32/del32 polymorphism was distributed as follows: ins/ins: 333 (80.6%); ins/del: 73 (17.7%) and del/del: 7 (1.7%). The genotype distribution did not deviate significantly from Hardy-Weinberg equilibrium (p=0.21). Baseline characteristics are shown in Table 1. The patient characteristics for the different genotype groups were similar at the start of dialysis, except antihypertensive medication use. Patients homo- or heterozygous for the deletion allele used more antihypertensive medications (p=0.01). From the 413 patients included, 225 (55%) had the CCR5 ins/ins genotype and low hsCRP levels, 108 (26%) the CCR5 ins/ins genotype and high hsCRP levels, 55 (13%) the CCR5Δ32 polymorphism and low hsCRP levels and 25 (6%) the CCR5Δ32 polymorphism and high hsCRP levels.

Table 1: Baseline characteristics

	N=413
Gender: male	253 (61.3)
Age (year)	62 (50-71)
Caucasian	379 (91.8)
Hemodialysis	277 (67.1)
Peritoneal dialysis	136 (32.9)
Primary kidney disease	
Diabetes mellitus	75 (18.2)
Glomerulonephritis	48 (11.6)
Renal vascular disease	76 (18.4)
Other	214 (51.8)
Cardiovascular disease	144 (34.9)
Diabetes mellitus	105 (25.4)
Smoking	
Never	120 (29.2)
Former	194 (47.2)
Current	97 (23.6)
DBP (mmHg)	83 (12.8)
SBP (mmHg)	150 (25.4)
Antihypertensive medication	356 (86.2)
Lipid lowering medication	121 (29.3)
hsCRP (mg/l)	5.1 (1.9-13.7)
hsCRP > 10 (mg/l)	133 (32.2)
Cholesterol (mmol/l)	5.0 (1.3)
Albumin (g/l)	32.5 (6.9)
Hemoglobin (g/dl)	11.0 (1.4)
GFR (ml/min)	4.2 (3.1)
Kt/V /week	2.3 (0.9)

Mortality and transplantation rates

Annual transition probabilities without CCR5 antagonist therapy are shown in Table 2. The probability of renal transplantation was lower in the patient group with CCR5 ins/ins genotype and systemic inflammation compared to the three other patient groups. Cardiovascular and non-cardiovascular mortality was higher in the patient group with CCR5 ins/ins genotype and systemic inflammation compared to the other patient groups. In the Markov model, pharmacological CCR5 blockade in this patient group improved survival and the probability of renal transplantation up to the level of patients with the CCR5Δ32 polymorphism and systemic inflammation (Table 2).

Cost-effectiveness

Parameters used for the analyses are shown in Table 3. Screening for the CCR5Δ32 polymorphism and treating patients with the CCR5 ins/ins genotype and systemic inflammation with pharmacological CCR5 blockade resulted in an average of 0.36 life years and 0.31 QALYs gained at an expense of €8,482 per patient compared to €1,863 per patient in the non-screening cohort (Table 4). Therefore, the incremental cost-effectiveness ratio (ICER) of the screen-and-treat strategy compared to not screening was €18,557 per life-year gained (LYG) and €21,896 per QALY gained. Results were similar without the model assumption that pharmacological CCR5 blockade improved patients' probability of renal transplantation, €18,494 per LYG and €24,642 per QALY gained.

As described, the unrelated future costs of dialysis and transplantation care due to improved survival were not included. The aforementioned increased survival of 0.36 life years in the genetically screened cohort indeed required considerable dialysis costs. These costs were only partly offset by a shift towards less costly renal transplantation care in these patients. In total, additional unrelated future costs were €6,720 per patient in the screening cohort. When these costs are included, the cost-effectiveness of the selective screen-and-treat strategy rose considerably to €37,400 per LYG and €44,127 per QALY gained, thus doubling the ICERs for these scenarios.

Table 2: Annual transition probabilities (95% CI) in the four CCR5Δ32 polymorphism and inflammation status groups, without treatment with pharmacological CCR5 blockade.[19]

	CCR5 ins/ins, No inflammation (n=225)	**CCR5 ins/ins, High inflammation * (n=108)**	**CCR5 Δ32, No inflammation (n=55)**	**CCR5 Δ32, High inflammation (n=25)**
Transplantation	10.9% (8.9-13.4%)	5.1% (3.0-8.4%)	11.2% (7.4-16.8%)	11.8% (6.4-21.5%)
Transplantation graft failure	2.2% (1.2-4.0%)	2.2% (1.2-4.0%)	2.2% (1.2-4.0%)	2.2% (1.2-4.0%)
Cardiovascular mortality	4.3% (3.2-5.7%)	9.5% (6.8-13.1%)	4.1% (2.3-7.4%)	4.0% (1.5-10.3%)
Non-cardiovascular mortality	4.4% (3.3-5.8%)	9.7% (7.0-13.4%)	4.5% (2.6-7.8%)	7.8% (4.0-15.1%)

* In the genotyping strategy of the economic model, patients with the CCR5 ins/ins and high inflammation status received CCR5 antagonists; thereby increasing transplantation rates and reducing mortality rates up to the level of patients with the CCR5Δ32 polymorphism and high inflammation status.

Table 4: Cost-effectiveness in the base-case analysis

	Costs	**Life years**	**QALY**
Standard care	€ 1,863	5.71	4.36
Screen-and-treat strategy	€8,482	6.07	4.67
Screen-and-treat strategy (no Tx effect)	€8,460	6.07	4.63

Cost-effectiveness	**Cost per LYG**	**Cost per QALY gained**
Screen-and-treat strategy	€18,557	€21,896
Screen-and-treat strategy (no Tx effect)	€18,494	€24,642

Table 3: Parameters used in the analyses

Variable	*Baseline value ± SD*	*Reference*
Costs		
Discounting rate for costs	4%	[43, 63]
Related costs *		
Genetic screening test	€50 ± 13	[36]
CRP screening test	€21 ± 5	
Drug costs Maraviroc (per year)	€5,057 ± 1,264	[37]
Transplantation graft failure	€4,581 ± 1,145	[39]
Cause of death		
Myocardial ischemia and infarction	€2,448 ± 612	[38]
Cardiac failure/ fluid overload/ pulmonary oedema	€4,529 ± 1,132	[38]
Cardiac arrest	€2,448 ± 612	[38]
Cerebro-vascular accident	€5,753 ± 1,438	[38]
Mesenteric infarction	€3,550 ± 888	[38]
Hyperkalaemia	€1,224 ± 306	[38]
Cause unknown or cause uncertain **	€3,469 ± 867	[38]
Non-cardiovascular mortality	€2,316 ± 579	[39]
Unrelated future costs *		
ESRD care costs		
Hemodialysis year 1	€84,825 ± 21,206	[31]
Hemodialysis later years	€80,482 ± 20,121	[31]
Peritoneal dialysis year 1	€65,706 ± 16,427	[31]
Peritoneal dialysis later years	€60,985 ± 15,246	[31]
Transplantation year 1	€52,199 ± 13,049	[31]
Transplantation later years	€10,440 ± 2,610	[31]
Health effects		
Discounting rate for health effects	1.5%	[43, 63]
Quality of Life		
Hemodialysis	0.71 ± 0.275	[31]
Peritoneal dialysis	0.75 ± 0.256	[31]
Transplantation	0.86 ± 0.133	[33]
Mortality and transplantation probabilities	See table 1	[19]
Therapy effectiveness (Relative Risk)		
All-cause mortality	0.61	[19]
Cardiovascular mortality	0.41	[19]
Non-cardiovascular mortality	0.80	[19]
Renal transplantation	2.41	[19]

* In the absence of data on standard deviations for costs, we assumed 25% of the mean.
** weighted average of all cardiovascular mortality causes.

Sensitivity & threshold analyses

Results of the probabilistic sensitivity analysis are shown in Figure 2, demonstrating the uncertainty around the cost-effectiveness estimates of the screen-and-treat strategy. The increase in cost-effectiveness as well as the uncertainty around these estimates due to including unrelated future costs is evident. In Figure 2, the solid dot denotes the base-case outcome (using the most likely parameter estimates) while the inner and outer ellipses denote the 50% and 90% probability intervals, respectively, around this base-case estimate. Univariate sensitivity analyses showed that the main drivers of the cost-effectiveness of the screen-and-treat strategy were the costs of pharmacological CCR5 blockade and the effectiveness of pharmacological CCR5 blockers to reduce mortality. The cost-effectiveness was relatively insensitive to plausible variations of the other parameters. These two main parameters were further explored in a threshold analysis, shown in Figure 3. The red line in this figure denotes the base-case assumptions for drug effectiveness and treatment costs. With decreasing therapy costs and increasing therapy effectiveness, cost-effectiveness of the screen-and-treat strategy improved. With the costs of pharmacological CCR5 blockade at the base-case level of €5,057 per year or €421 per month, a RR for all-cause mortality of 0.82 or lower would cause the cost-effectiveness of the screen-and-treat strategy to be €50.000 or less per QALY gained. If the costs of CCR5 blockers drop, even a modest effectiveness in reducing inflammation-driven mortality would result in a treatment strategy that is good value for money.

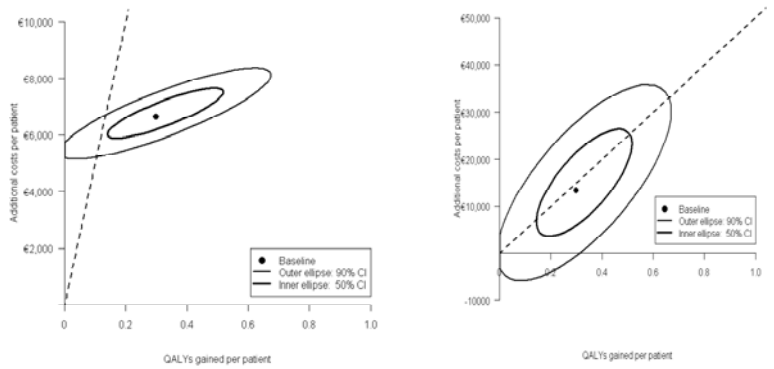

Figure 2a and b: Cost-effectiveness of the screen-and-treat strategy. Figure a (left panel): excluding unrelated future costs (ESRD-care costs). Figure b (right panel): including unrelated future costs. Dotted line denotes the willingness-to-pay threshold for one QALY at €50,000 [34].

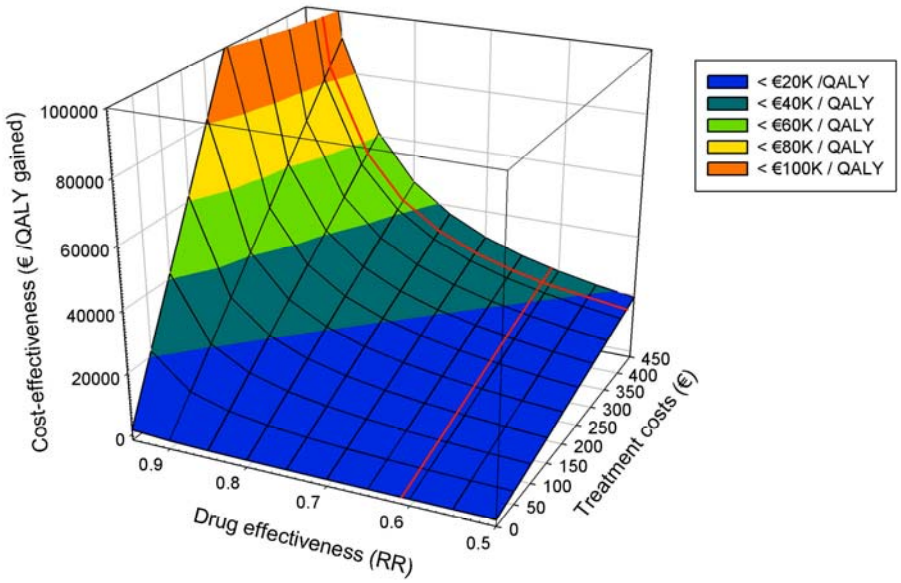

Figure 3: Threshold analysis on the influence of CCR5 blocking therapy costs and effectiveness on the cost-effectiveness of a screen-and-treat strategy. The red lines denote the base case parameters for drug effectiveness and treatment costs.

Discussion

Our study analyzed the potential cost-effectiveness of screening for the CCR5Δ32 polymorphism and selectively treating dialysis patients with the CCR5 ins/ins genotype and systemic inflammation with pharmacological CCR5 blockers. It was shown that such a strategy could be incorporated in a potentially cost-effective genetic screen-and-treat program.

Observational studies in which a genetic polymorphism is associated with a well-characterized functional phenotype can be considered as a type of clinical trial, with randomization at conception, referred to as Mendelian randomization.[4-6] Following this approach, we investigated the presumption that in an analogous manner, pharmacological CCR5 blockade could lead to better survival in ESRD patients and estimated the cost-effectiveness of a genetic screen-and-treat strategy based on this strategy. We used data from a genetic association study in ESRD patients. In this study an association with better survival was found in incident dialysis patients with systemic inflammation carrying the CCR5Δ32 genotype, which was replicated in a Swedish ESRD cohort, hereby showing the robustness of these findings. Moreover, since the number of patients in the CCR5Δ32 groups was small, we did in the previous study an analysis on the two cohorts combined, leading to the same results.[19] The presence of the CCR5Δ32 polymorphism, leading to a less functional receptor,[14] was used as a naturalistic form of pharmacologically blocking the CC-chemokine 5 receptor. This approach was used recently in Cholesterol Ester Transfer Protein (CETP) inhibition, identifying alleles which lead to reduced CETP levels and activity.[47] Other cost-effectiveness assessments of potential pharmacologic interventions have previously been performed, for example in cardiovascular disease and polypill therapy.[48] Considering the ACCE (analytic validity, clinical validity, clinical utility and ethical, legal and social issues) model framework for enhancing the evaluation of genetic tests, our study adds to the second C by providing cost-effectiveness data that supports clinical utility.[49, 50]

A long-standing controversy in health-economics is whether unrelated future costs should be included in cost-effectiveness analyses.[40, 41, 51, 52] Dialysis treatment is expensive and associated with a high cost per QALY gained.[31, 53] As dialysis is required lifelong, the cost-effectiveness of therapies in ESRD patients have been said to be driven more by dialysis costs than by the costs and benefits of the intervention under consideration itself.[54] Our analysis

confirms these earlier findings and underscores the relevance of the debate by calculating that inclusion of dialysis and renal transplant care costs doubles the incremental cost-effectiveness ratio of the screen-and-treat strategy. Several studies in ESRD patients did not include the future costs of ESRD-care,[55-57] while others analysed therapies both with and without future costs.[58-60] By excluding ESRD-costs in the main analysis but including them in a separate analysis our results can be widely compared. The cost-effectiveness with inclusion of future ESRD-costs were comparable to other studies focusing on systemic anticoagulation,[60] hyperphosphataemia,[59] secondary hyperparathyroidism,[58] and anaemia.[61]

In addition to adherence to guidelines for pharmacoeconomic research as possible within the constraints of novel pharmacogenetic screening programs,[22] the present study had two major strengths: 1. the analyses considered hard end points, mortality and renal transplantation; 2. most primary data used in the pharmacoeconomic analysis, such as costs, quality of life estimates and efficacy data, were derived from a single prospectively followed dialysis cohort (NECOSAD). These strengths enhanced the clinical relevance and analytical robustness of the study findings. Although cost data used in our study were specific for the Netherlands, chronic kidney disease (CKD) care costs such as dialysis costs have been reported to fall within a narrow range despite considerable variation in country of study, methodology and imputed costs.[53] Country specific variations in drug costs and discounting rates have been accounted for in sensitivity analyses.

An important aspect of our study is the notion that equivalence between genetic effects and associated pharmacologic effectiveness is not a given fact. For example, a discordance has been described between the genetic effect of familial hypercholesterolaemia and the effectiveness of statin treatment on cardiovascular mortality.[46] The explanation for this discrepancy lies in the fact that genetic factors, as opposed to pharmacologic interventions, cause life-long differences in risk factors.[46] Genetic factors are also not affected by traditional sources of uncertainty in clinical effectiveness, such as therapy compliance. Indeed, sensitivity analyses showed that the cost-effectiveness was highly influenced by the concordance between the genetic association and pharmacological effectiveness. Still, while the true effectiveness of pharmacological CCR5 blockade in ESRD patients on mortality is not (yet) known, this study, in particular the threshold analysis, provides valuable information for future

clinical trials in this field. In this context, the threshold analysis showed that even modest pharmacological effectiveness would result in a treatment strategy that is good value for money. A similar approach has recently been taken in analyzing the potential cost-effectiveness of alternative treatments for CKD patients resistant to ACE inhibitors due to ACE (I/D) polymorphisms.[36] Finally, the robustness of the cost-effectiveness estimate depends on whether or not pharmacologically blocking CCR5 is safe in ESRD patients. However, treating HIV-infected ESRD patients with a CCR5 antagonist seemed safe and no dose adjustments were necessary.[62] The next research step could be conducting an observational cohort study in HIV-infected ESRD patients, to compare cardiovascular morbidity or mortality or surrogate endpoints such as intima media thickness, among users and non-users of CCR5 blocker therapy.

In conclusion, we evaluated the potential cost-effectiveness of pharmacologically blocking the CCR5 receptor in inflamed dialysis patient with the CCR5 ins/ins genotype, and found it to be similar to existing treatment modalities for dialysis patients. Recently CCR5 blockade has indeed become feasible in humans. Our data suggest that, from an economic point of view, it would be worthwhile to study whether pharmacological blockade of CCR5 has therapeutic and economical benefits in dialysis patients with persistent inflammation. Our study is an illustration of the potential of genetic studies in drug-development programs, as a new source of Mendelian randomized evidence from an observational setting.

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and facilitate genetic and genomic studies to the clinical benefit of the renal patient.

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Chapter 7

SUMMARY AND FUTURE PERSPECTIVES

Summary

In end stage renal disease (ESRD) and type 2 diabetes atherosclerosis and chronic inflammation are important factors in the high morbidity and mortality seen in these populations.[1-7] Since therapies proven to be successful in the general population have thus far been disappointing in patients with ESRD,[8-10] and since atherosclerosis is nowadays considered a chronic inflammatory disease,[11, 12] the inflammatory process is an area of increased interest in these patients.

In this process of inflammation and atherosclerosis chemokines play an important role.[13, 14] Chemokines can be classified into four major categories (C, CC, CXC and CX3C). The CC chemokines and their receptors have been widely implicated in atherosclerosis.[15, 16] Among these, the CC-chemokine receptor 5 (CCR5) is expressed on T cells, monocytes/ macrophages, smooth muscle cells and endothelial cells.[17, 18] These cells are involved in the chronic inflammatory state present in insulin resistance, type 2 diabetes, atherosclerosis and uremia.[3, 7, 11, 19] Several polymorphisms have been described for CCR5. The CCR5Δ32 genetic variant is located on the chromosome 3p21 and consists of a 32-basepair deletion in the open reading frame. It effectively results in functional CCR5 deficiency by absence of CCR5 membrane expression.[20] Different human association studies showed that CCR5Δ32 is associated with better outcome in patients with a high risk for atherosclerotic cardiovascular disease, and in renal transplant recipients, probably by modulation of the inflammatory response in these conditions.[21-24] In animal studies using mouse models the role of CCR5 has been verified by both pharmacological receptor antagonism and genetic deletion.[25-34] The mechanisms by which CCR5 and CCR5 deficiency contribute to chronic inflammation and atherosclerosis are believed to be due to their effects on immune cell migration and response.[14, 35] Notably, in mice CCR5 deficiency was associated with a reduction in Th1 type immune response.[25, 28] Moreover, in animal models CCR5 deficiency modulates monocyte recruitment in atherosclerotic lesions and is associated with improved plaque stability.[14, 16, 27]

Taken together, these data suggest that CCR5 might be involved in the accelerated atherosclerosis in chronic inflammatory conditions like ESRD and type 2 diabetes. Substantiation of this role would be clinically relevant, as CCR5 might provide a novel target for intervention, with, moreover, also

practical feasibility as pharmacological blockers of CCR5 are already available and in use in anti-HIV therapy.[36]

The studies in this thesis therefore, were aimed at exploring the possible role of CCR5 in the increased (cardiovascular) mortality in the chronic inflammatory, atherosclerotic conditions of ESRD and type 2 diabetes, and at exploring its potential as a possible target for intervention.

In chapter 2 we demonstrated that the presence of CCR5Δ32 is associated with better survival in patients with type 2 diabetes.[37] These data are in line with the impact of the CCR5Δ32 in several other populations as mentioned above. The protective effect of the CCR5Δ32 is allegedly due to a dysfunctional CCR5 leading to modulation of inflammatory responses. Unfortunately, in this population we had no data available on the severity of the inflammatory state or on possible differences in inflammatory pathways between the genotypes to support such a mechanism of protection.

In ESRD CRP is associated with overall and cardiovascular mortality, demonstrating the impact of inflammation on outcome in this high risk population.[38] In chapter 3 we demonstrated that the CCR5Δ32 genotype attenuates the adverse effects of an inflammatory state on overall and cardiovascular mortality in ESRD, in two independent cohorts of patients with ESRD, from the Netherlands and Sweden.[39] These data support the clinical impact of a gene-environment interaction, the inflammatory state being the environmental factor with an adverse effect on outcome, that is however blunted by the genetic factor CCR5Δ32, possibly by genetically mediated CCR5 deficiency. Support for such a mechanism is provided in chapters 4 and 5. In chapter 4 we report on a reduced Th1 type immune response as represented by decreased TNF-α levels in patients with ESRD carrying the CCR5Δ32 genotype as compared to non-carriers.[40] In chapter 5, subsequently, we describe for the first time in a functional, human study the possible modifying effect of the CCR5Δ32 genotype on the Th1/Th2 disequilibrium of atherosclerotic inflammation. Stimulated CD4+ and CD8+ T cells of patients with one or two CCR5Δ32 alleles show an increased Th2 type phenotype base on their intracellular cytokine profile. These differences in immune response may be involved in the impact of CCR5Δ32 on outcome in ESRD.

As these data support the relevance of CCR5 as a novel target for intervention in the chronic inflammatory process in ESRD, in chapter 6 we performed a simulation study on the potential cost-effectiveness of CCR5Δ32 screening and pharmacological CCR5 blockade in dialysis patients. In this study we used the documented effects of genetically determined CCR5 deficiency in ESRD to estimate the clinical benefits of pharmacological CCR5 blockade in this population. This estimate was used for a pharmaco-economic analysis on the effects of pharmacological CCR5 blockade in ESRD. Based on this simulation study we conclude that, from an economic point of view, it would be worthwhile to study whether pharmacological blockade of CCR5 has therapeutic and economical benefits in dialysis patients with persistent inflammation and the CCR5 ins/ins genotype. This study is an illustration of the potential of genetic studies in drug-development programs, as a new source of Mendelian randomized evidence from an observational setting.[41]

The studies described in this thesis show that CCR5 is of importance in the chronic inflammatory, atherosclerotic process seen in patients with type 2 diabetes and in patients with ESRD and that chemokine pathways, especially CCR5 could be a novel target for intervention that could theoretically have considerable impact on outcome. Our data show, that in patients with ESRD with chronic inflammation a dysfunctional CCR5 reduced the annual probability on mortality from approximately 10% to 5%.[39, 41] Whereas, obviously, these figures should not straightforwardly be extrapolated to the results of a pharmacological intervention, nevertheless, it is clear that the contribution of CCR5 in the mortality in this patient group, and hence its potential as a target for intervention, is substantial.

Several animal studies using mouse models suggested a role for blockade of the CCR5 pathway as a therapy in atherosclerotic, inflammatory conditions.[29-31] To date, only one small molecule antagonist of CCR5, Maraviroc, has cleared the many hurdles needed to permit licensing for clinical usage and is used in the treatment of HIV infection.[36, 42] For cardiovascular or other inflammatory diseases no human, intervention studies are available. The reason for this most likely is the complexity of the role of chemokines and chemokine receptors in the inflammatory process seen in cardiovascular disease.

At present, the chemokine system consists of 50 chemokines and more than 19 chemokine receptors. They play a role in the multistep leucocyte adhesion cascade and they regulate a wide range of processes.[43] A characteristic of the majority of chemokine receptors is their high affinity for multiple ligands. This implies that the same ligand can cause different biological effects depending on the type of chemokine receptor expressed on target cells.[35, 44] Also, depending on the anatomical site or the physiological circumstances chemokines can act differently and they display a considerable synergy.[13, 44] Recently it was demonstrated that chemokines are able to form oligomeric structures, mainly dimers and that the biological effect depends on the oligomerization.[13, 44] This all leads to the notion that the activity of chemokines can be specific to a distinct cell type, site and/or phase in plaque progression. For example, CCR5 is thought to be more important in the late stages of plaque development.[25, 27]

Interfering in the CCL2/CCR2, CCL5/CCR5, CX3CL1/CX3CR1, CXCL8/CXCR2 pathways could provide promising therapeutic targets for atherosclerotic disease, as supported by different animal models.[44] When focussing on CCR5 ligands several mouse and human tissue studies underscore a link with atherosclerotic disease. For example, knock-down of CCL5 expression on vascular smooth muscle reduces neointimal thickening and macrophage infiltration in ApoE-/- mouse models;[45] blocking CCL5 function was found to lessen progression of atherosclerotic plaque in LDLr-/- mice;[26] disruption of CCL5 and platelet factor PF-4 led to a reduction in atherosclerotic lesions in hyperlipidemic mice;[46] Expression of CCL3, CCL4 and CCL5 has been detected in human arteries and atherosclerotic plaques.[17, 47, 48]

Taking into account the specific effects of the different chemokines and chemokine receptors in the atherogenic cell recruitment, the potential role of blocking multiple components of the chemokine system simultaneously for prevention and treatment of atherosclerosis has been advocated. For example, targeting three chemokine-receptor systems, CCL2/CCR2, CCL5/CCR5, CX3CL1/CX3CR1, could be a promising strategy.[33, 49]

Possible adverse effects of chemokine intervention for prevention and therapy of atherosclerosis have to be considered as well. Because the chemokine system is an integral part of the immune system shutting down it constituents might be accompanied by an increased risk of unwanted immunological side effects. For example, CCR5Δ32 seem to be associated

with infection with the West Nile virus and tickborne encephalitis.[50, 51] Moreover, considering the nature of atherosclerosis as a chronic inflammatory condition, tolerability and safety issues related to long term use should be addressed. Delivering tailored therapy to the patient categories likely to benefit most could be a way to favourable affect the risk-benefit ratio of treatment. Based on our results, for instance, it would be logical to take into account inflammatory status, and genotype of the patient. In ESRD the category likely to benefit from CCR5 blockade, accordingly, would be patients with the CCR5 ins/ins genotype and signs of systemic inflammation.[39, 41]

In conclusion, a significant amount of evidence can be found supporting the therapeutic potential of certain chemokine-chemokine receptor blockade. Given the fact that CCR5 seems to play a crucial role in cardiovascular disease and given the fact that Maraviroc is currently the only pharmacological chemokine blocker available as a clinically validated drug on the market, albeit for a completely different condition, provides a significant advantage for targeting CCR5 over the other chemokine-receptors. However, numerous issues should be resolved. Given the high risk for cardiovascular disease in patients with HIV-infection,[52] it could be fruitful, in this respect, to systematically document inflammatory status and cardiovascular complications in HIV positive patients treated with Maraviroc.

Future perspectives

Current therapy in cardiovascular disease in the general population and in high risk populations like type 2 diabetes and ESRD has mainly targeted classical risk factors such as elevated blood presssure and lipid levels, and smoking. More recently, proteinuria has become a target for intervention as well. Although this strategy is generally effective, there remains an urgent need for innovative therapeutic approaches, in particular in high-risk groups such as ESRD and type 2 diabetes, as apparently, current therapy fails to reduce the excessively high risk for atherosclerotic complications in these conditions. Given the extensive role of the innate and adaptive immune responses in atherosclerosis, targeting its cellular constituents seems a valuable approach for attenuating the disease process. As systemic modulation of immune responses can severely compromise host defence, a dissection of the cellular mechanisms that are involved in atherogenesis will allow targeted interventions that might minimize the unwanted effects of broad-spectrum treatments. Because chemokines and their receptors are key mediators in the immune response seen in atherogenesis, targeting these pathways is promising. However, the precise mechanism of the different chemokine pathways in the establishment and progression of atherosclerotic disease and the therapeutic potential of blocking chemokine pathways have yet to be elucidated. In this complex process multiple gene-environmental interactions play an important role. Genetic association studies can be used to discover new pathways, and indicate their impact on the burden of disease in human populations. The associations should lead to further mechanistic studies both in animal and human models. Eventually randomized controlled trials with hard end points in well-defined patient populations are needed. To facilitate this expensive and time consuming process, genetic differences between persons leading to well characterized functional phenotypes can be used as a heuristic strategy to explore individualised treatment possibilities and to simulate clinical trial data.

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NEDERLANDSE SAMENVATTING

Ondanks grote vooruitgang in de behandeling van hart- en vaatziekten door behandeling van risicofactoren zoals hypertensie en hypercholesterolemie blijven cardiovasculaire ziekten een belangrijke oorzaak van ziekte en overlijden wereldwijd. Met name ook bij patiënten met diabetes mellitus type 2 en bij patiënten met eindstadium nierfalen zijn cardiovasculaire aandoeningen veel voorkomend. In deze laatste groep speelt nog mee dat behandelingen die succesvol zijn gebleken in de algemene populatie, zoals bijvoorbeeld cholesterol verlaging, minder succesvol zijn. Dit onderstreept het belang van het verder ontrafelen van het onderliggende pathofysiologische proces van hart- en vaatziekten, speciaal bij patiënten met eindstadium nierfalen om uiteindelijk nieuwe therapieën te ontwikkelen.

Tegenwoordig wordt het onderliggende proces van hart- en vaatziekten gezien als een ontstekingsreactie. Aan het begin van dit proces, ook wel bekend als het 'response to injury proces', is er sprake van activatie en dysfunctie van endotheelcellen in de bloedvaten. Deze cellen raken geactiveerd door omgevingsfactoren. Ook genetische factoren spelen hierbij een rol in een zogenaamde gen-omgeving interactie. De endotheel-dysfunctie geeft aanleiding tot vorming van adhesie-moleculen, vrijkomen van chemokinen en cytokinen en influx van ontstekingscellen zoals monocyten, T-cellen en andere ontstekingscellen in de subendotheliale ruimte. Als gevolg van deze ontstekingsreactie in de subendotheliale ruimte ontstaat allereerst een zogenaamde ‘fatty streak’. Verdere ontsteking leidt tot de ontwikkeling van atherosclerotische plaques. Deze plaques kunnen uiteindelijk leiden tot klinische symptomen door bijvoorbeeld bloedvatvernauwing of afsluiting.

Bij de onderliggende ontstekingsreactie kan men onderscheid maken in de aangeboren immuunreactie waarbij monocyten/ macrofagen, natural killer T-cellen en dendritische cellen een rol spelen en specifieke afweer waarbij B- en antigeen specifieke T-cellen een rol spelen. Verschillende subklassen van T-cellen zijn hierbij van belang. Dit zijn voornamelijk CD4+ T-cellen en in mindere mate CD8+ T-cellen. De CD4+ T-cellen kunnen differentiëren in T-helper 1 (Th1) cellen, T-helper 2 (Th2) cellen, T-helper 17 (Th17) cellen en regulatoire T-cellen. Th1 cellen produceren interferon gamma (IFN-γ) als belangrijkste cytokine. Activatie van Th1 cellen leidt tot tumor necrose factor alpha (TNF-α) secretie. De Th1 type immuunreactie wordt beschouwd als pro-inflammatoir en pro-atherogeen. Van Th2 cellen en de cytokinen, interleukine 4 (IL-4), interleukine 5 (IL-5) en interleukine 10 (IL-10), wordt

aangenomen dat ze, in ieder geval deels, een anti-inflammatoire en anti-atherogene rol spelen.

In dit ontstekingsproces spelen ook chemokinen een belangrijke rol. Chemokinen binden aan chemokine-receptoren, die aanwezig zijn op ontstekingscellen zoals T-cellen, monocyten/ macrofagen en spelen een rol bij de verplaatsing van deze cellen naar ontstekingsgebieden. In muizenstudies is gebleken dat uit de grote chemokine familie voornamelijk de CC-chemokinen en hun receptoren betrokken zijn bij atherosclerose. Een van deze CC-chemokine-receptoren is de CC-chemokine 5 receptor (CCR5). Van deze chemokine-receptor zijn verschillende genetische varianten bekend waaronder het zogenaamde CCR5Δ32 polymorfisme. Deze variant leidt uiteindelijk tot CCR5 deficiëntie door afwezigheid van expressie aan het celoppervlak bij homozygoten en verminderde expressie bij heterozygoten. In de Europese populatie is ongeveer 15% heterozygoot en 1-2% homozygoot voor deze genetische variant. Bij HIV-positieve patiënten is aangetoond dat CCR5Δ32 geassocieerd is met resistentie tegen infectie met HIV hetgeen aantoont dat de mutatie inderdaad tot functionele veranderingen leidt. Verder werd in patiëntengroepen met een hoog risico op hart- en vaatziekten de CCR5Δ32 mutatie geassocieerd met een betere uitkomst, hoewel hier ook tegenstrijdige berichten over zijn. Als verklaring voor deze gunstige effecten van de aanwezigheid van CCR5Δ32 wordt gedacht aan een modulatie van de immuunreactie. In muizen lijkt CCR5 deficiëntie tot een verminderde Th1 type immuunreactie te leiden en is sprake van een meer stabiele atherosclerotische plaque.

Deze zaken bij elkaar opgeteld, samen met het feit dat er bij patiënten met eindstadium nierfalen sprake is van een chronische ontstekingsreactie, maken dat CCR5 mogelijk een geschikte kandidaat is om het ontstekingsproces, dat bij de nierpatiënt tot hart- en vaatziekten leidt, te beïnvloeden. Het interessante van CCR5 is dat er een geneesmiddel op de markt is dat CCR5 blokkeert. Dit geneesmiddel wordt gebruikt bij de behandeling van HIV-infectie. Door de beschikbaarheid van dit middel zijn er praktische mogelijkheden om te onderzoeken of CCR5 blokkade effect heeft op hart- en vaatziekten bij de mens. In dit proefschrift bespreken we daarom epidemiologische en functionele consequenties van CCR5Δ32 in eindstadium nierfalen en diabetes mellitus type 2.

In het tweede hoofdstuk hebben we de hypothese getoetst of de aanwezigheid van CCR5Δ32 bij patiënten met diabetes mellitus type 2 geassocieerd is met overleving. We hebben hiervoor gebruik gemaakt van een cohort patiënten met diabetes mellitus type 2 uit de regio Zwolle (ZODIAC cohort) dat werd gevolgd gedurende een langere periode. De aanwezigheid van de CCR5Δ32 genetische variant bleek gerelateerd te zijn aan betere overleving. We veronderstellen dat dit te maken heeft met verschillen in beloop van ontstekingsprocessen tussen patiënten met en zonder de CCR5Δ32 variant. Echter omdat er in dit cohort geen gegevens beschikbaar waren over ontstekingsactiviteit konden we deze veronderstelling niet toetsen.

In een volgende studie, bij patiënten met eindstadium nierfalen, was het wel mogelijk om de rol van ontstekingsactiviteit mee te nemen. Bij deze patiënten was namelijk het C-reactive protein (CRP) gemeten. CRP is een marker van ontsteking; bij patiënten met eindstadium nierfalen is een verhoogd CRP geassocieerd met mortaliteit in het algemeen en cardiovasculaire mortaliteit hetgeen wijst op een belangrijke rol van ontstekingsactiviteit in de sterfte bij deze patiënten, in het bijzonder aan hart- en vaatziekten. Het zou zo kunnen zijn dat in dit ontstekingsproces een dysfunctionele CCR5 receptor een gunstige invloed heeft door verminderde migratie van ontstekingscellen. In het derde hoofdstuk hebben we daarom in een Nederlands en een Zweeds patiëntencohort met eindstadium nierfalen onderzocht of aanwezigheid van de CCR5Δ32 genetische variant een dempend effect heeft op de relatie tussen een verhoogd CRP en overleving. Dit bleek inderdaad het geval te zijn; patiënten met de CCR5Δ32 genetische variant en een verhoogd CRP hadden een vergelijkbare overlevingskans als patiënten zonder verhoogd CRP. Bij patiënten met een verhoogd CRP, zonder de CCR5Δ32 genetische variant was de overleving significant slechter. Dit is een voorbeeld van een duidelijke gen-omgeving interactie: de genetische variant beschermt tegen de ongunstige effecten van ontsteking.

In hoofdstuk 4 en 5 wordt gezocht naar de mechanismen van het beschermende effect. Hiervoor is het van belang om te weten dat pro-inflammatoire cytokinen geassocieerd zijn met slechte uitkomst in patiënten met eindstadium nierfalen en dat een immuunreactie van het type Th1 en de bijbehorende cytokinen pro-atherogeen zijn, hetgeen betekent dat ze vaatschade kunnen veroorzaken. Uit muizenstudies is bekend, zoals boven reeds vermeld, dat CCR5 deficiëntie, zowel genetisch als door

farmacologische blokkade leidt tot een immuun respons met meer type Th2 dan Th1 kenmerken zoals minder pro-inflammatoire cytokine productie. Om na te gaan of de CCR5Δ32 variant gepaard gaat met minder cytokine release als reactie op ontsteking hebben we in een cohort patiënten met eindstadium nierfalen gekeken of de CRP, TNF-α, IL-6 en IL-10 spiegels verschilden in dragers en niet-dragers van de CCR5Δ32 genetische variant. Het bleek inderdaad zo te zijn dat de TNF-α spiegels significant lager waren bij patiënten met aanwijzingen voor systemische ontsteking blijkend uit een verhoogd CRP als er sprake was van de CCR5Δ32 genetische variant. In het vijfde hoofdstuk hebben we geprobeerd om de hypothese dat de CCR5Δ32 genetische variant ertoe leidt dat de immuunreactie sterkere Th2 dan Th1 kenmerken vertoont en daardoor vaatschade kan remmen verder te onderbouwen. Hiertoe stimuleerden we T-cellen van dragers en niet-dragers van de CCR5Δ32 genetische variant met eindstadium nierfalen om zo de ontstekingsreactie na te bootsen. Vervolgens bepaalden we het intracellulaire cytokine profiel van deze cellen en vergeleken dit met elkaar. Hieruit bleek dat zowel CD4+ als CD8+ T-cellen van dragers van de CCR5Δ32 genetische variant na stimulatie een meer uitgesproken Th2 type immuunreactie laten zien. Dit is de eerste maal dat bewezen wordt dat CCR5Δ32 bij de mens effect heeft op de eigenschappen van de immuunrespons. Deze ‘gunstige’ immuunreactie zou een verklaring kunnen zijn voor het beschermende effect van CCR5Δ32 bij patiënten met eindstadium nierfalen.

De ontwikkeling van nieuwe farmacologische therapieën is een kostbare aangelegenheid zowel in tijd als in geld. Om deze reden is er behoefte aan alternatieven om dit proces te vergemakkelijken. Observatiestudies, waarbij genetische varianten met een goed gekarakteriseerd, functioneel fenotype worden bestudeerd, zouden hierbij kunnen helpen. Dergelijke studies kunnen namelijk worden beschouwd als een 'real life' simulatie van een klinische trial met randomisatie bij de conceptie (ook wel aangeduid als 'Mendeliaanse randomisatie'). Op grond van dit principe hebben we naar aanleiding van de bevindingen uit hoofdstuk 3 een simulatiestudie uitgevoerd naar de kosteneffectiviteit van screenen op aanwezigheid van CCR5Δ32 en vervolgens farmacologisch blokkeren van de CCR5 receptor bij patiënten met eindstadium nierfalen. In deze studie toonden we aan dat het screenen op CCR5Δ32 en het farmacologisch blokkeren van CCR5 kosteneffectief is bij patiënten met eindstadium nierfalen. Deze studie is een

illustratie van de bruikbaarheid van genetische associatiestudies bij de ontwikkeling van nieuwe farmacologische therapieën.

De studies in dit proefschrift onderstrepen het belang van CCR5 in het chronische ontstekingsproces dat leidt tot vaatschade bij patiënten met eindstadium nierfalen en ondersteunen de veronderstelling dat interventie in het chemokine systeem, speciaal blokkering van CCR5, een therapeutische optie zou kunnen zijn. Uit diverse muizenstudies waarbij gekeken werd naar vaatschade, is gebleken dat blokkade van CCR5 gunstige effecten heeft. Het is bemoedigend dat er voor humaan gebruik een CCR5 antagonist beschikbaar is, gebruikt als antiviraal middel bij HIV-positieve patiënten. Er zijn echter geen data voorhanden waarin dergelijke geneesmiddelen worden gebruikt ter bescherming tegen hart- en vaatziekten bij de mens. Een mogelijke oorzaak hiervoor is de zeer complexe rol die chemokinen en chemokine-receptoren spelen bij hart- en vaatziekten. Zo hebben chemokinen een hoge affiniteit voor verschillende receptoren: dit betekent dat een chemokine meerdere effecten kan sorteren afhankelijk van het type receptor waaraan het bindt. Daarnaast is het zo dat chemokinen ook verschillende effecten kunnen sorteren afhankelijk van de fysiologische omstandigheden en dat er een bepaalde mate van synergie tussen chemokinen bestaat. Bovendien kan de structuur van een chemokine veranderen en daarmee ook het biologische effect. Door dit alles kan het effect van chemokinen en van chemokine-receptoren verschillen per celtype, lokalisatie en fase van ontstekingsreactie. Van CCR5 wordt bijvoorbeeld aangenomen dat dit van groter belang is in de latere stadia van plaque ontwikkeling dan in eerdere stadia.

Naast bovengenoemde overwegingen betreffende de werkzaamheid moet natuurlijk ook rekening gehouden worden met eventueel nadelige effecten van interventie in het chemokine systeem. Omdat chemokinen en chemokine-receptoren een onderdeel vormen van het immuunsysteem zou blokkering ervan een verhoogde kans op infecties kunnen geven. Het is bijvoorbeeld bekend dat aanwezigheid van CCR5Δ32 genetische variant de kans op infectie met het 'West Nile virus' verhoogt en ook de kans op het krijgen van een bepaalde vorm van encephalitis. Als laatste moeten ook tolerantie en veiligheidsaspecten meegenomen worden die gerelateerd zijn aan het langdurig gebruik van geneesmiddelen die worden ingezet bij chronische ziekten zoals hart- en vaatziekten. Om de kosten-baten-ratio

gunstig te laten uitvallen verdient het aanbeveling om te zoeken naar patiëntengroepen die het meeste baat zouden kunnen hebben bij een dergelijke therapie; een en ander vraagt dus om therapie op maat.

Zoals uit dit proefschrift blijkt kunnen genetische associatiestudies helpen bij het uiteindelijk ontwikkelen van therapie op maat. Dergelijke studies kunnen worden gebruikt om pathofysiologische mechanismen op het spoor te komen, kunnen een indicatie geven van mogelijk te verwachten effecten bij medicamenteuze interventie en kunnen helpen om die patiënten te identificeren die speciaal baat hebben bij een betreffende therapie. Tevens kan uit dit proefschrift geconcludeerd worden dat het chronische ontstekingsproces dat leidt tot vaatschade en meer specifiek de centrale rol die chemokinen en chemokine-receptoren hierin spelen vanuit zowel pathofysiologisch als therapeutisch oogpunt een veel belovend terrein van onderzoek is bij patiënten met hart- en vaatziekten, speciaal bij patiënten met eindstadium nierfalen en bij patiënten met diabetes mellitus type 2 die ondanks de huidige therapieën een hoog risico lopen om aan hart- en vaatziekten te overlijden.

DANKWOORD

Dankwoord

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Zoals Marieke, Nienke en Douwe zeiden: "Hocus pocus pief poef paf, papa's proefschrift is nu af."